Questionnaire-based survey on the clinical management of canine leishmaniosis in the Madrid region (central Spain)

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Abstract

This paper describes a questionnaire designed to gain information on how veterinarians clinically manage canine leishmaniosis (CanL) in the Madrid region (central Spain). The present survey is one of several similar questionnaire-based surveys conducted within the framework of the project EDEN (Emerging Diseases in a changing European eNvironment). The questionnaire sought to obtain data regarding the main clinical manifestations observed, the diagnostic methods used and the preventive measures recommended. Its Spanish version was sent by post to veterinary practitioners within the study area in two lots, one sent out in December 2006 and the other in March 2007. Only 174 of the 760 questionnaires sent were completed and returned (reply rate of 23%). Among the completed questionnaires, clinics differed widely in terms of features such as the habitats of the dogs (urban, peri-urban or rural) and patient volumes. Clinics attending dogs from peri-urban/rural habitats reported more suspected ($p<0.001$), confirmed ($p=0.001$) and newly diagnosed ($p=0.001$) cases/year than clinics providing service to a city clientele alone. According to the veterinary practitioners, skin lesions, lymphadenomegaly and weight loss were commonly observed, although these signs are not specific to CanL. Signs described to be of high diagnostic value were epistaxis and kidney disease. All the veterinarians polled reported that a suspicion of *Leishmania infantum* infection was confirmed by at least a serological method; the immunofluorescence antibody test (IFAT) being the technique most used. To prevent the disease, most vets recommended topical synthetic pyrethroids applied as impregnated collars or spot-ons. It is observed that despite considerable progress is being made in clinical management and controlling the disease, in Madrid Region its incidence continues to increase.

37 Keywords (3-6 items): canine leishmaniosis, Madrid, clinical management,
38 veterinarians, questionnaire.

1. Introduction

Leishmaniosis is a widespread zoonotic disease that is endemic in the Mediterranean basin. The disease, caused by the parasite *Leishmania infantum* (Nicolle, 1908), is transmitted to humans and animals by blood-sucking phlebotomine sand flies. The dog is the main domestic reservoir for human infection by *L. infantum*. Infected dogs, whether clinically healthy or sick, are infectious for phlebotomine sand flies (Molina et al., 1994). This determines that infection prevalence in endemic areas is considerably higher than the rate of apparent clinical illness (Baneth et al., 2008).

Clinical canine leishmaniosis (CanL) shows a wide spectrum of clinical signs and clinical-pathological abnormalities because of the many pathogenic mechanisms involved and the particular immune response produced in the host (Solano-Gallego et al., 2009). The main clinical signs of CanL are one or more of the following: weight loss, lethargy, muscular atrophy, anaemia, lymphadenomegaly, splenomegaly, epistaxis, diarrhoea, hepatic and/or renal disorders, ocular lesions, polyarthritis, onychogryphosis and skin lesions (Baneth et al., 2008; Miró et al., 2008; Paltrinieri et al., 2010).

This broad spectrum of clinical signs makes the diagnosis of CanL especially challenging (Solano-Gallego et al., 2009). Thus, the diagnosis of CanL requires an integrated approach based on epidemiological data, an exhaustive clinical examination, quantitative serological techniques, routine diagnostic tests to detect clinical-pathological abnormalities, and microscopy observation of *Leishmania* amastigotes in tissues (Miró et al., 2008; Paltrinieri et al., 2010).

In the absence of a fully effective vaccine against CanL, protecting dogs with topical insecticides is the best way to reduce the spread of *Leishmania* in endemic areas, as both laboratory and field studies have shown (Maroli et al., 2001; Gavgani et al., 2002;

63 Reithinger et al., 2004; Foglia Manzillo et al., 2006; Maroli et al., 2010; Otranto et al.,
64 2010). The most widely tested modes of application have been dog collar, spot-on and
65 spray formulations (Molina et al., 2001; Mencke et al., 2003; Mencke et al., 2005;
66 Molina et al., 2006; Miró et al., 2007; Otranto et al., 2007). The correct use of topical
67 formulations is crucial to prevent sand fly bites and thus avoid the transmission of CanL
68 to dogs (Solano-Gallego et al., 2009).

69 Estimates of CanL seroprevalence reported for Spain range from 3.7% for Orense
70 province in the northwest corner of the country (Amusátegui et al., 2004) to 34.6% for
71 Málaga province on the south coast (Morillas et al., 1996). In the Madrid region,
72 significant increases in the seroprevalence of CanL and the densities of its vectors
73 (*Phlebotomus perniciosus* and *Phlebotomus ariasi*) have been detected with respect to
74 surveys conducted 17 and 15 years ago ~~(Gálvez et al., 2010a; Gálvez et al., 2010b)~~. In
75 the Madrid region, vector densities were significantly higher in 2008 than densities
76 reported in 1991 (Gálvez et al., 2010a). Moreover, the average prevalence of CanL was
77 estimated to have increased 1.54-fold in the Madrid region, from 5.25% in 1992 to 8.1%
78 in 2006-2007 (Gálvez et al., 2010b).
79 Despite the reported spread of CanL, veterinary approaches to tackle *L. infantum*
80 infection vary widely. The aim of the present survey was to determine how veterinarians
81 manage dogs with CanL in the Madrid region with special attention paid to the main
82 clinical manifestations observed, diagnostic methods used and preventive measures
83 recommended. The opinions of the vets regarding trends in the incidence of the disease
84 were also sought by the questionnaire.

85 2. Materials and methods

2.1. Study area

The study was performed in Madrid, central Spain (40º22´N and 3º43´W) where CanL is endemic. The altitude of this region varies from 491 to 2400 m. The climate and vegetation are typically Mediterranean, with dry, hot summers and maximum rainfall recorded in autumn and spring. The survey was conducted under the EDEN project (www.eden-fp6project.net), whose aim is to identify and catalogue European ecosystems and environmental conditions that determine the spatial and temporal distributions and dynamics of several pathogens, including *L. infantum*.

2.2. Data collection

As part of the Leishmaniosis project of EDEN (EDEN-LEI), a questionnaire was designed to be administered to veterinarians by all the EDEN-LEI partners (Fig. 1). For the present purpose, this questionnaire (translated from French into Spanish), an explanatory letter and a stamped addressed envelope were posted to veterinary practitioners within the study area (Madrid city and surrounding area) in two lots, one posted in December 2006 ~~(640 questionnaires)~~ and the other in March 2007 ~~(120 questionnaires)~~. The first lot (consisted of 640 vets) was sent by Madrid Veterinary Association (AMVAC) to its members. Due to confidentiality, AMVAC did not provide us the vet addresses, so we cannot contact with non responding veterinarians. In the second lot (consisted of 120 clinics) we resent questionnaires only to veterinarians of the study area proposed for EDEN project (band spanning northeast to southwest). Addresses from the second lot were obtained from the web page www.qdq.com. In total, 760 questionnaires were sent. Additionally, 61 non responding veterinarians from the second lot were contacted by phone to explain the importance of filling out the questionnaire. The questionnaire was designed to obtain data on: the number of dogs examined weekly at the veterinary clinic, the number of suspected/confirmed and newly

111 diagnosed CanL cases in the past year, the frequency of CanL clinical signs detected
112 and their diagnostic value, the diagnostic method of choice, preventive measures
113 recommended, whether the veterinarians thought dogs with CanL had been infected or
114 not in their usual habitat and the opinions of the veterinarians on the trends in CanL
115 infection produced over the past ten years. The locations of veterinary clinics were
116 geocoded using the geocoding tool of the GIS software on a street map layer for the
117 Madrid province updated in 2004 (Fig. 2).

118 2.3. Statistical Analysis

119 Categorical variables were described as percentages. Chi-squared and Fisher's exact
120 tests were used to analyze associations. Suspected, confirmed and newly diagnosed
121 cases of CanL were categorized as fewer or more than 20 cases per year for
122 comparisons between urban and peri-urban/rural dog habitats. Veterinarians were
123 classified into two groups according to the extent of agreement observed between
124 suspected and confirmed cases and then Pearson Chi-Square test was made between
125 clinical signs and the two groups. Frequencies (1-3) and the diagnostic value (1-3) of
126 clinical signs were also categorized into one variable as the sum of the two variables (2-
127 6) to determine which clinical signs were the most relevant according to veterinarians.

128 3. Results

129 3.1. Reply rate and veterinary clinic clientele

130 Only 174 out of 760 questionnaires sent were returned completed to give a reply rate of
131 only 23%. Non responding veterinarians from the first lot had a low response rate: 120
132 out of 640 questionnaires sent were returned, reply rate 19%. In contrast, the second lot
133 had an acceptable reply rate: 54 out of 120 questionnaires sent were returned, reply rate
134 45%. Most of the clientele of the veterinarians who filled in the questionnaire was

comprised of dogs living in urban (46%) or peri-urban (49%) areas compared to rural (5%). In most practices, the number of dogs examined weekly was over 20 (76%), although some vets reported 11-20 (21%) or even 4-10 dogs (2%) seen per week.

3.2. Epidemiology

Table 1 presents the epidemiological variables reported by the vets completing the questionnaire on CanL and divided by veterinarian clientele. The numbers of cases of suspected CanL observed in the past year were >50 (16%), 21-50 (16%), 11-20 (22%), 6-10 (19%), 1-5 (25%) and 0 (1%). Among suspected cases, the numbers confirmed were >50 (3%), 21-50 (16%), 11-20 (21%), 6-10 (15%), 1-5 (37%) and 0 (2%). Numbers of newly diagnosed cases recorded in the past year were >50 (1%), 21-50 (8%), 11-20 (16%), 6-10 (19%), 1-5 (42%) and 0 (7%). Thus, more suspected ($p<0.001$) and confirmed cases ($p=0.001$) were observed in peri-urban/rural clinics, and these clinics also reported more newly diagnosed cases at 13% (>20 cases/year) compared to 5% (>20 cases/year) reported for urban clinics ($p=0.001$) (Fig. 2).

While many veterinarians in peri-urban/rural areas thought that the CanL infections they had dealt with had been acquired in the area where the dogs lived (92%), some veterinarians attending urban dogs thought they had been infected outside the city (35%). The trends in CanL noted over the past ten years were an increased incidence reported by 34% of the vets, a decrease according to 39% and no change according to 18%.

3.3. Frequency and diagnostic value of clinical signs

Table 2 provides the frequencies and diagnostic value of clinical signs and clinical-pathological abnormalities of CanL on which the veterinarians based their suspicion of infection. When we looked at the relevance of each clinical sign (frequency + diagnostic

value in Table 2), lymphadenomegaly, exfoliative dermatitis and weight loss emerged as most relevant, whereas hyperthermia, lethargy, anaemia and ocular lesions as being of least importance. Unspecific signs such as cutaneous lesions or lymphadenomegaly were highly described, whereas less frequently observed signs like epistaxis or kidney disease were thought to be of great diagnostic value.

There were no differences (p-values ranged from 0.132 to 0.824) in the clinical signs indicated by veterinarians claiming good agreement (76%) between their suspected and confirmed cases and those who reported poor agreement (24%).

3.4. Diagnostic methods

All veterinarians used at least one serological diagnostic method, whether quantitative (IFAT or ELISA) or qualitative (immunochromatography using a dipstick), to confirm a case of suspected CanL. Half the vets used a single method (IFAT, 85%; ELISA, 6% or dipstick, 9%), and the other half based their final diagnosis on the results of 2 (38%), 3 (8%), 4 (3%) or up to 5 different tests (2%). In rank order, the diagnosis techniques mostly used were IFAT (91%), dipstick (29%), microscopy observation (26%), PCR (14%) and ELISA (11%). Diagnosis was carried out by a private laboratory in 83% of the clinics, at the veterinary clinic in 12% and at a departmental veterinary laboratory in 5%.

3.5. Preventive measures

In this section of the questionnaire, the veterinarians were asked about the topical insecticides they generally recommend to their dog owners. 99% of vets polled recommended topical insecticides to avoid sand fly bites. Collars (92%) or spot-ons (93%) were the most recommended formulations, while sprays (21%) and shampoos (1%) were less recommended. Only one vet (1%) considered that topical insecticides

were ineffective. Moreover, 95% of the vets confirmed that all commercially available topical insecticides are labelled to indicate protection against sand fly bites.

4. Discussion

The data on CanL provided by the responding practitioners varied widely in terms of the factors dog habitat and volume of patients. More (13%) veterinarian clinics whose clientele was comprised of the owners of dogs from both rural and peri-urban areas reported seeing more >20 cases of CanL/year compared to urban clinics (5%). In a survey on CanL carried out in southeast Spain, also based on the EDEN questionnaire, it was also found that most veterinarians seeing more than 20 cases of CanL per year were those with clinics in rural areas (de Ybañez et al., 2009). The incidence of CanL detected here was higher in peri-urban than urban areas despite a lower volume of patients (Fig. 2).

Veterinarians considered that a case of CanL was of local origin if dog lived at least during the transmission period in the area of their practice. ~~Moreover t~~The majority of veterinarians examining rural or peri-urban dogs (92%) considered that the cases of CanL detected were of a local origin. In contrast, only 65% of vets providing service to urban dogs supported the local origin hypothesis. In a prior study conducted in the Madrid region, the influence of environmental variables on vector densities was observed to be lower in urban areas (Gálvez et al., 2010a; Gálvez et al., 2011). It is well documented that the risk of CanL transmission is higher in rural or periurban environments where dogs and sand flies come into close contact (Martínez-Cruz et al., 1990; Nieto et al., 1992; Celaya, 1993; Castañeda et al., 1999; Solano-Gallego et al., 2006).

Despite poor agreement among the practitioners regarding the trends produced in CanL in the last decade in the Madrid region, emerging trends for this disease have been

recently reported for this area (Gálvez et al., 2010a; Gálvez et al., 2010b). The emerging trends are supported by our questionnaire results. In the Madrid region we have recently proved that veterinary clinics registering a higher incidence of CanL appeared closer to vector foci (Gálvez et al, 2011). In Italy, the northward spread of CanL has been described (Maroli et al., 2008; Morosetti et al., 2009).

The clinical signs described as most frequent and of greatest diagnostic value were cutaneous lesions, lymphadenomegaly and weight loss. Signs described as not so frequents but of high diagnostic value were epistaxis and kidney disease. In effect, the cutaneous and/or systemic manifestations of CanL have substantially remained unchanged over the years (Baneth et al., 2008; de Ybañez et al., 2009; Solano-Gallego et al., 2009; Paltrinieri et al., 2010). In endemic areas, a single sign compatible with disease should justify the use of a diagnostic test to confirm the suspicion of CanL (Miró and Molina, 2006). The management of CanL should include differential diagnosis to rule out other vector-borne diseases (e.g., ehrlichiosis, babesiosis) or diseases producing cutaneous lesions (e.g., autoimmune diseases, endocrine disorders) (Solano-Gallego et al., 2009). Veterinarians should also keep in mind that many dogs with CanL can have concomitant disorders linked to the immunocompromised state of the animal or to another vector transmitted disease (Cortese et al., 2006; Foglia Manzillo et al., 2008). Moreover, the zoonotic potential of this disease must be considered by the clinicians.

All the vets surveyed based their suspicion of *L. infantum* infection on at least a serological method; IFAT being the technique most often used. The most popular diagnostic approach in the EDEN questionnaires administered to veterinarians in southeast Spain and northern Italy was also serological testing (de Ybañez et al., 2009; Morosetti et al., 2009). An accurate diagnosis of CanL requires the use of quantitative

serological tests (Miró et al., 2008; Solano-Gallego et al., 2009) supported by an exhaustive clinical examination and/or the detection of clinical-pathological abnormalities (Miró and Molina, 2006). Although a conclusive diagnosis is the direct observation of the parasite in stained smears obtained from lymph nodes, bone narrow or a skin citology, microscopy observation only shows a low sensitivity since it is dependant on parasite load and observer skills (Alvar et al., 2004). PCR is not usually used for diagnostic purposes, because the presence of *Leishmania* DNA in blood or other tissues only means that these dogs harbour the infection but not necessarily present the clinical disease (Solano-Gallego et al., 2009). Otherwise, real-time PCR is an advanced technique that can quantify *Leishmania* loads in tissues of infected dogs which is important for diagnosis as well as for follow-up of treated dogs (Pennisi et al., 2005; Francino et al., 2006; Manna et al., 2008).

The vets polled valued the topical application of synthetic pyrethroids as a preventive measure and collars and spot-ons were the formulations most recommended. In a further two EDEN questionnaires in Spain and Italy, synthetic pyretroids given as spot-ons or impregnated collars were also the preventive measures most recommended (de Ybañez et al., 2009; Morosetti et al., 2009). In effect, preventing sand fly bites is probably the best method of interrupting the spread of *L. infantum* (Miró et al., 2008) and the concerted effort of vets to make dog owners aware of the importance of the use of topical insecticides during the whole sand fly season could reduce the incidence of leishmaniosis both in humans and dogs (Gavgani et al., 2002; Podaliri Vulpiani et al., 2009; Maroli et al., 2010).

Despite the fact that today dogs are better cared for and that progress is being made in clinical management and the topical insecticides available, we have recently observed a

moderate expansion in CanL in the Madrid region (Gálvez et al., 2010b; Gálvez et al., 2011). Further studies should try to explain this expansion.

5. Conclusion

The veterinarians with practices in the Madrid region surveyed here seem to be well informed and aware of CanL. ~~It is clear that clinical management of the disease and control measures have improved in the past few years ago.~~ However, recent evidence suggests the broad distribution and expansion of CanL. The control of CanL in endemic areas should be based on the widespread use of measures targeted at preventing new infections. These measures include the use of topical insecticides, good clinical management, adequate care of dogs and chemotherapy. Although considerable progress is being made in controlling this disease, in endemic areas its incidence continues to increase.

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References

Alvar, J., Cañavate, C., Molina, R., Moreno, J., Nieto, J., 2004. Canine leishmaniasis. Advances in parasitology 57, 1-88.

Amusátegui, I., Sainz, A., Aguirre, E., Tesouro, M.A., 2004. Seroprevalence of *Leishmania infantum* in northwestern Spain, an area traditionally considered free of leishmaniasis. Annals of the New York Academy of Sciences 1026, 154-157.

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.

Castañeda, R., Carpio, I., Asensio, A., Alberdi, J.C., García, S., Domínguez, T., Moreno, S., 1999. Estudio de seroprevalencia de la leishmaniosis canina en perros censados en Madrid. Consulta de difusión veterinaria. 7, 836-841.

Celaya, C., 1993. Estudio epidemiológico descriptivo de la leishmaniasis canina en un entorno periurbano de la ciudad de Madrid. Veterinaria en Madrid. 5, 13-15.

Cortese, L., Pelagalli, A., Piantedosi, D., Mastellone, V., Manco, A., Lombardi, P., Ciaramella, P., Avallone, L., 2006. Platelet aggregation and haemostatic response in dogs naturally co-infected by Leishmania infantum and Ehrlichia canis. J Vet Med A Physiol Pathol Clin Med 53, 546-548.

de Ybañez, R.R., del Rio, L., Martínez-Carrasco, C., Segovia, M., Cox, J., Davies, C., Berriatua, E., 2009. Questionnaire survey on Canine Leishmaniosis in southeastern Spain. Veterinary parasitology 164, 124-133.

Foglia Manzillo, V., Oliva, G., Pagano, A., Manna, L., Maroli, M., Gradoni, L., 2006. Deltamethrin-impregnated collars for the control of canine leishmaniasis: evaluation of the protective effect and influence on the clinical outcome of

Leishmania infection in kennelled stray dogs. Veterinary parasitology 142, 142-145.

Foglia Manzillo, V., Pagano, A., Guglielmino, R., Gradoni, L., Restucci, B., Oliva, G., 2008. Extranodal gammadelta-T-cell lymphoma in a dog with leishmaniasis. Veterinary clinical pathology / American Society for Veterinary Clinical Pathology 37, 298-301.

Francino, O., Altet, L., Sánchez-Robert, E., Rodríguez, 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.

Gálvez, R., Descalzo, M.A., Guerrero, I., Miró, G., Molina, R., 2011. Mapping the current distribution and predicted spread of the leishmaniosis sand fly vector in the Madrid region (Spain) based on environmental variables and expected climate change Vector borne and zoonotic diseases (Larchmont, N.Y) doi:10.1089/vbz.2010.109.

Gálvez, R., Descalzo, M.A., Miró, G., Jiménez, M.I., Martín, O., Dos Santos-Brandao, F., Guerrero, I., Cubero, E., Molina, R., 2010a. Seasonal trends and spatial relations between environmental/meteorological factors and leishmaniosis sand fly vector abundances in Central Spain. Acta tropica 115, 95-102.

Gálvez, R., Miró, G., Descalzo, M.A., Nieto, J., Dado, D., Martín, O., Cubero, E., Molina, R., 2010b. Emerging trends in the seroprevalence of canine leishmaniosis in the Madrid region (central Spain). Veterinary parasitology 169, 327-334.

Gavgani, A.S., Hodjati, M.H., Mohite, H., Davies, C.R., 2002. Effect of insecticide-impregnated dog collars on incidence of zoonotic visceral leishmaniasis in Iranian children: a matched-cluster randomised trial. Lancet 360, 374-379.

Manna, L., Reale, S., Vitale, F., Picillo, E., Pavone, L.M., Gravino, A.E., 2008. Real-time PCR assay in Leishmania-infected dogs treated with meglumine antimoniate and allopurinol. Vet J 177, 279-282.

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 dogs. Journal of the American Veterinary Medical Association 236, 1200-1206.

Maroli, M., Mizzon, V., Siragusa, C., D'Oorazi, A., Gradoni, L., 2001. Evidence for an impact on the incidence of canine leishmaniasis by the mass use of deltamethrin-impregnated dog collars in southern Italy. Medical and veterinary entomology 15, 358-363.

Maroli, M., Rossi, L., Baldelli, R., Capelli, G., Ferroglio, E., Genchi, C., Gramiccia, M., Mortarino, M., Pietrobelli, M., Gradoni, L., 2008. The northward spread of leishmaniasis in Italy: evidence from retrospective and ongoing studies on the canine reservoir and phlebotomine vectors. Trop Med Int Health 13, 256-264.

Martínez-Cruz, M.S., Martínez-Moreno, A., Martínez-Moreno, F.J., Martínez-Gómez, F., Hernández-Rodríguez, S., 1990. Epidemiología de la leishmaniosis canina en Córdoba. Revista Ibérica de Parasitología 50, 1-7.

Mencke, N., Volf, P., Volfova, V., Stanneck, D., 2003. Repellent efficacy of a combination containing imidacloprid and permethrin against sand flies (*Phlebotomus papatasi*) in dogs. Parasitology research 90 Suppl 3, S108-111.

Mencke, N., Volf, P., Volfova, V., Stanneck, D., Miró, G., Gálvez, R., Mateo, M., Montoya, A., Molina, R., 2005. Repellent efficacy of an imidacloprid/permethrin spot-on against sandflies (*Phlebotomus papatasi, P. perniciosus and Lutzomyia longipalpis*). In, Proceedings of the Eighth International Symposium on Ectoparasite Pets, Hannover, Germany, p. 61.

Miró, G., Cardoso, L., Pennisi, M.G., Oliva, G., Baneth, G., 2008. Canine leishmaniosis--new concepts and insights on an expanding zoonosis: part two. Trends in parasitology 24, 371-377.

Miró, G., Gálvez, R., Mateo, M., Montoya, A., Descalzo, M.A., Molina, R., 2007. Evaluation of the efficacy of a topically administered combination of imidacloprid and permethrin against *Phlebotomus perniciosus* in dog. Veterinary parasitology 143, 375-379.

Miró, G., Molina, R., 2006. Leishmaniosis canina: Manejo clínico y situación actual en España

Molina, R., Amela, C., Nieto, J., San-Andrés, M., González, F., Castillo, J.A., Lucientes, J., Alvar, J., 1994. Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*. Transactions of the Royal Society of Tropical Medicine and Hygiene 88, 491-493.

Molina, R., Lohse, J.M., Nieto, J., 2001. Evaluation of a topical solution containing 65% permethrin against the sandfly (*Phlebotomus perniciosus*) in dogs. Vet Ther 2, 261-267.

Molina, R., Miró, G., Gálvez, R., Nieto, J., Descalzo, M.A., 2006. Evaluation of a spray of permethrin and pyriproxyfen for the protection of dogs against *Phlebotomus perniciosus*. The Veterinary record 159, 206-209.

373 Morillas, F., Sánchez Rabasco, F., Ocaña, J., Martín-Sánchez, J., Ocana-Wihelmi, J.,
374 Acedo, C., Sanchís-Marín, M.C., 1996. Leishmaniosis in the focus of the
375 Axarquia region, Malaga province, southern Spain: a survey of the human, dog,
376 and vector. Parasitology research 82, 569-570.
377 Morosetti, G., Bongiorno, G., Beran, B., Scalone, A., Moser, J., Gramiccia, M.,
378 Gradoni, L., Maroli, M., 2009. Risk assessment for canine leishmaniasis
379 spreading in the north of Italy. Geospatial health 4, 115-127.
380 Nieto, C.G., Navarrete, I., Habela, M., Hernández-Rodríguez, S., 1992. Seroprevalence
381 of canine leishmaniasis around Cáceres,Spain. Preventive Veterinary Medicine
382 13, 173-178.
383 Otranto, D., de Caprariis, D., Lia, R.P., Tarallo, V., Lorusso, V., Testini, G., Dantas-
384 Torres, F., Latrofa, S., Diniz, P.P., Mencke, N., Maggi, R.G., Breitschwerdt, E.,
385 Capelli, G., Stanneck, D., 2010. Prevention of endemic canine vector-borne
386 diseases using imidacloprid 10% and permethrin 50% in young dogs: a
387 longitudinal field study. Veterinary parasitology 172, 323-332.
388 Otranto, D., Paradies, P., Lia, R.P., Latrofa, M.S., Testini, G., Cantacessi, C., Mencke,
389 N., Galli, G., Capelli, G., Stanneck, D., 2007. Efficacy of a combination of 10%
390 imidacloprid/50% permethrin for the prevention of leishmaniasis in kennelled
391 dogs in an endemic area. Veterinary parasitology 144, 270-278.
392 Paltrinieri, S., Solano-Gallego, L., Fondati, A., Lubas, G., Gradoni, L., Castagnaro, M.,
393 Crotti, A., Maroli, M., Oliva, G., Roura, X., Zatelli, A., Zini, E., 2010.
394 Guidelines for diagnosis and clinical classification of leishmaniasis in dogs.
395 Journal of the American Veterinary Medical Association 236, 1184-1191.

Pennisi, M.G., Reale, S., Giudice, S.L., Masucci, M., Caracappa, S., Vitale, M., Vitale, F., 2005. Real-time PCR in dogs treated for leishmaniasis with allopurinol. Veterinary research communications 29 Suppl 2, 301-303.

Podaliri Vulpiani, M., Iannetti, L., Di Mattia, T., Dalla Villa, P., 2009. *Leishmania infantum* in a Central Italy dog shelter: Retrospective study of serologic reactivity during a 4-year period in a confined dog population subjected to preventive and therapeutic treatment. Veterinary parasitology 160, 190-197.

Reithinger, R., Coleman, P.G., Alexander, B., Vieira, E.P., Assis, G., Davies, C.R., 2004. Are insecticide-impregnated dog collars a feasible alternative to dog culling as a strategy for controlling canine visceral leishmaniasis in Brazil? International journal for parasitology 34, 55-62.

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.

Solano-Gallego, L., Llull, J., Osso, M., Hegarty, B., Breitschwerdt, E., 2006. A serological study of exposure to arthropod-borne pathogens in dogs from northeastern Spain. Veterinary research 37, 231-244.

EPIDEMIOLOGICAL VARIABLES	Veterinarians clientele N (%)	
	Urban	Peri-urban & Rural
Cases of suspected CanL in the past year		
Fewer than 20	67 (85)	49 (53)
More than 20	12 (15)	43 (47)
Cases of confirmed CanL in the past year		
Fewer than 20	67 (92)	63 (71)
More than 20	6 (8)	26 (29)
Newly diagnosed cases recorded in the past year		
Fewer than 20	69 (95)	77 (87)
More than 20	4 (5)	11 (13)
Dogs were infected in the area of your practice?		
No	24 (35)	8 (8)
Yes	46 (65)	84 (92)
Trends in CanL over the past ten years		
Increase	19 (27)	41 (46)
Decrease	38 (53)	30 (34)
No change	14 (20)	18 (20)

Table 1. Epidemiological variables reported by the vets completing the questionnaire on CanL and divided by veterinarians clientele

420

	FREQUENCY (%)			DIAGNOSTIC VALUE (%)			RELEVANCE
CLINICAL SIGNS	**Rarely**	**Frequently**	**More than frequently**	**Low**	**Medium**	**High**	**Median (p25-p75)**
Weight loss (*) (+)	7	66	27	18	43	39	4 (4-5)
Lethargy	47	44	9	69	24	7	3 (2-4)
Splenomegaly	31	49	13	41	36	23	4 (3-4)
Hyperthermia	92	6	2	95	3	2	2 (2-2)
Epistaxis (+)	51	35	13	13	28	59	4 (3-5)
Alopecia (*)	17	46	37	35	38	28	4 (3-5)
Exfoliative dermatitis (*) (+)	5	45	50	18	38	44	5 (4-6)
Onycogryphosis	31	50	19	35	40	26	4 (3-5)
Ulcerative dermatitis (*) (+)	19	57	25	25	38	38	4 (3-5)
Lymphadenomegaly (*) (+)	2	39	59	7	21	72	6 (5-6)
Ocular lesions	44	45	8	43	36	21	3 (2-4)
CLINICAL-PATHOLOGICAL ABNORMALITIES	**Rarely**	**Frequently**	**More than frequently**	**Low**	**Medium**	**High**	**Median (p25-p75)**
Kidney disease (+)	31	53	15	24	41	35	4 (3-5)
Anaemia	37	56	7	49	41	10	3 (2-4)

421 (*) Signs described as “frequently” or “more than frequently” by more than 70 percent of vets. (+) Signs
422 considered to be of “high” or “medium” diagnostic value by more than 70 percent of vets.

423 **Table 2.** Clinical signs and clinical-pathological abnormalities reported by the vets completing
424 the questionnaire on CanL

425

426 **Fig. 1.** EDEN questionnaire on the clinical management of CanL mailed to veterinarians
427 in the Madrid region in 2006-2007.
428

Fig. 2. Incidence of canine leishmaniosis reported by veterinarians in the Madrid region calculated according to the number of dogs examined per week.

Table 1

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Table 1. Epidemiological variables reported by the vets completing the questionnaire on CanL and divided by veterinarians clientele

Table 2

	FREQUENCY (%)			DIAGNOSTIC VALUE (%)			RELEVANCE
CLINICAL SIGNS	**Rarely**	**Frequently**	**More than frequently**	**Low**	**Medium**	**High**	**Median (p25-p75)**
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(*) Signs described as “frequently” or “more than frequently” by more than 70 percent of vets. (+) Signs considered to be of “high” or “medium” diagnostic value by more than 70 percent of vets.

Table 2. Clinical signs and clinical-pathological abnormalities reported by the vets completing the questionnaire on CanL

Figure1.1
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QuickTime™ et un décompresseur TIFF (non compressé) sont requis pour visionner cette image.

Questionnaire on Canine Leishmaniosis

1. Details of the vet:

Name: .....
Clinic: .....
Address: ..... Town: .....
Telephone: .....

2. Where do your clients come from?:
Urban areas ☐ Rural ☐ Mixed ☐ Other (please specify) ☐
.....

3. How many dogs do you examine per week?
0 ☐ 1-3 ☐ 4-10 ☐ 11-20 ☐ more than 20 ☐

4. How many dogs with suspected leishmaniosis have you seen in the past 12 months?
0 ☐ 1-5 ☐ 6-10 ☐ 11-20 ☐ 21-50 ☐ more than 50 ☐

5. On which clinical signs do you base your suspicion?

Sign	Frequency of observation			Importance given within the diagnostic suspicion (+ to +++)
	Rarely	Frequently	More than frequently	
Weight loss				
Lethargy				
Anaemia				
Hyperthermia				
Epistaxis				
Alopecia				
Exfoliative dermatitis				
Onycogryphosis				
Ulcerative dermatitis				
Lymphadenomegaly				
Ocular lesions				
Kidney disease				
Splenomegaly				
Other				

If you indicate Other, please specify:
.....

Figure 1.2
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6. How many confirmed cases of canine leishmaniosis have you seen in the past 12 months?
0 ☐ 1-5 ☐ 6-10 ☐ 11-20 ☐ 21-50 ☐ more than 50 ☐

7. How many of these confirmed cases were new?
0 ☐ 1-5 ☐ 6-10 ☐ 11-20 ☐ 21-50 ☐ more than 50 ☐

8. How were these cases confirmed?
☐ Epidemiology
☐ Clinical examination
☐ Serology: ☐ IFI
☐ ELISA
☐ Rapid detection kit

☐ PCR
☐ Microscopy: ☐ Lymph node aspirate
☐ Bone marrow aspirate
☐ Skin biopsy
☐ Other (please indicate):
.....

9. Confirmation of these cases was conducted:
at your laboratory ☐,
at a private laboratory ☐
at a departmental veterinary laboratory ☐
other ☐, please indicate:.....

10. Do you think these dogs were infected in the area of your practice?
yes ☐ no ☐

11. Have you noticed any trends in your clinic over the past 10 years in the number of cases of canine leishmaniosis seen:
Yes, an increasing one ☐ Yes, a decreasing one ☐ No change ☐

12. What prevention method do you recommend to dog owners?
Collar ☐ Spot-on ☐ Spray ☐ Shampoo ☐ None ☐

13. If you do not recommend preventive measures, this is because:
You think they are ineffective ☐
They are too expensive ☐
You don't know where to get them ☐
You think the risk of leishmaniosis is low ☐
Other ☐, please indicate: .....

14. Do the preventive agents you recommend specifically indicate they have repellent effects on phlebotomine sand flies?:
yes ☐ no ☐

Figure 2 revised

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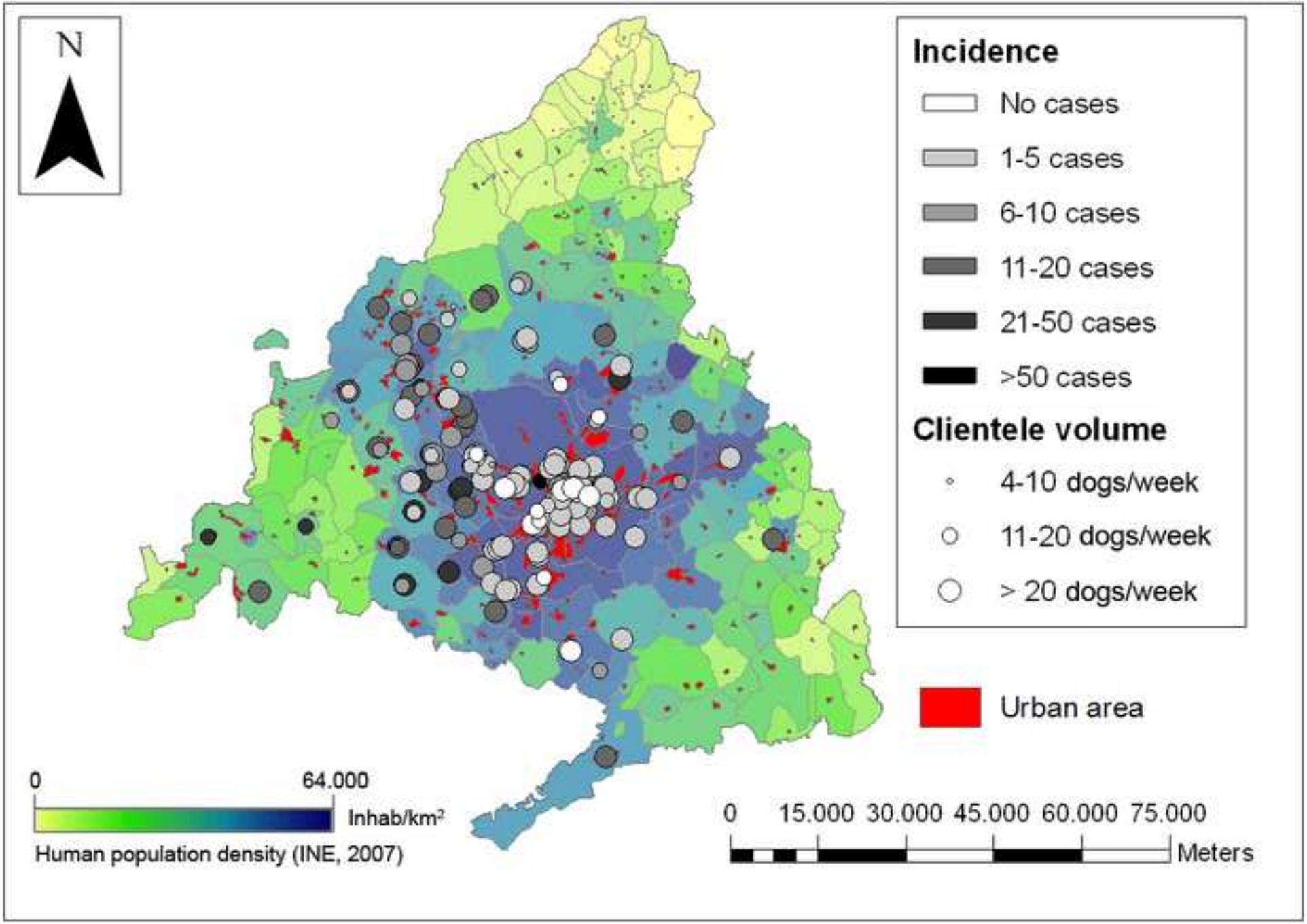

REVISION NOTE

Note: the line numbers are no longer the same since the order of the whole text has been modified. Changes in the second revision has been highlighted in green.

Reviewer number: 1

Referees comments on author's responses to questions raised on the article "Questionnaire-based survey on the clinical management of canine Leishmaniosis in the Madrid region (Central Spain)"

1) Referee's original question: is it really the case that "The cutaneous and/or systemic manifestations of CanL have substantially remained unchanged over the years" (page 9, lines 199-202).

Referee's subsequent comment: if what you mean is "that we have a lot of information about the clinical picture of this disease" do so, but this does not answer my question which relates to whether symptoms are different/similar now and some time before in Madrid or elsewhere.
This question is answered below with the nex one.
I suppose that to answer this question you need to refer to work on the subject published before 2008, which is the oldest article that you cite. In fact, the article quoted by Baneth and Aroch (2008), specifically says that clinical signs are variable admitting that only dermatitis is a common sign. So, in other words: are the relative proportions of clinical signs in dogs from Madrid similar/different to those occurring for example in Israel or Brazil or other parts of Spain?
The authors consider that the clinical picture of CanL is almost the same reported in the last two decades but maybe due to the advances in the good clinical management of this disease we have more information about the wide variety of clinical signs that may occur in a sick dog.

2) Referee's original question: "Authors mentioned several recent CanL and sandfly vector studies from the Madrid region but no attempt is made to relate the findings between those and the present study"

Referee's subsequent comment: Again, you don't answer the question raised; I am not asking you about the correlation between CanL cases and vector foci in the previous study by Galvez et al. instead, I would like to know if there is there a correlation between CanL incidence assessed in your questionnaire and CanL/vector foci in other previous studies in Madrid including the one by Galvez et al.
The second sentence in your response appears to indicate that there is indeed a correlation between questionnaire and previous studies. If this is the case then you should clearly say so in the text.
Authors added the following information (lines 207-210 of the revised manuscript): "The emerging trends are supported by our questionnaire results. In the Madrid region we have recently proved that veterinary clinics registering a higher incidence of CanL appeared closer to vector foci (Gálvez et al, 2011)."

3) Referee's original question: What do you mean by: "emerging trends for this disease have been recently reported for this area"? (page 9, lines 193-4) To what extent is this

supported by questionnaire results?

Referee's subsequent comment: ok, but this conclusion is based on previous studies and no attempt is made to reinforce it with questionnaire data so, personally, I think it should not be part of the conclusions of this article.
Authors indicated the correlation between questionnaire and previous studies with the information added in lines 207 to 210 of the revised manuscript.

4) Referee's original question: Table 1 is repeated twice. A table describing the frequency of responses which are presently in the text would be useful. Given that you used a GIS, what are the geographical and climatic features of high/low CanL incidence?

Referee's subsequent comment: ok, that's fine.

5) Referee's original question: One of the conclusions is that "clinical management and control of CanL have improved in the past few years" but no evidence is provided to support this. If control measures have improved, how do you justify saying (also in the conclusions) that incidence is higher now?

Referee's subsequent comment: ok, but like I said before this conclusion is based on previous studies and your study did not address this issue so in my opinion it should not be part of the conclusions of this article.
The sentence from the Conclusion: "It is clear that clinical management of the disease and control measures have improved in the past few years ago." has been removed (lines 260-261 of the revised manuscript) because as reviewer_1 said, this information is based on other studies.

Reviewer number: 2

The authors have addressed almost all of the questions in my review. However, their responses still need to be incorporated into the manuscript itself. And the spelling is Leishmaniasis.
For the denomination of parasitic diseases or infections, authors have followed the Standardized Nomenclature of Animal Parasitic Diseases (SNOAPAD) published in 1988 in *Veterinary Parasitology*. According to Kassai et al. (1988) the correct name of parasitic diseases is constructed solely by the suffix –osis, which is added to the stem of the name of the parasite taxon, formed from the nominative of the taxa. Authors would prefer to maintain the term "leishmaniosis" instead of "leishmaniasis".

For example "Line 70-72: What are the changes…." the answers are in the response to reviewer but haven't been added to the manuscript where they need to be.
Now this information has been included (lines 73 to 77 of the revised manuscript): "In the Madrid region, vector densities were significantly higher in 2008 than densities reported in 1991 (Gálvez et al., 2010a). Moreover, the average prevalence of CanL was estimated to have increased 1.54-fold in the Madrid region, from 5.25% in 1992 to 8.1% in 2006-2007 (Gálvez et al., 2010b)."

This is true for the comments in the original lines 95-96 (all of the details about the groups need to be added),

Now this information has been included (lines 100 to 105 of the revised manuscript): "The first lot (consisted of 640 vets) was sent by Madrid Veterinary Association (AMVAC) to its members. Due to confidentiality, AMVAC did not provide us the vet addresses, so we cannot contact with non responding veterinarians. In the second lot (consisted of 120 clinics) we resent questionnaires only to veterinarians of the study area proposed for EDEN project (band spanning northeast to southwest). Addresses from the second lot were obtained from the web page www.qdq.com."

line 116,
Now this information has been included (lines 130 to 133 of the revised manuscript): "Non responding veterinarians from the first lot had a low response rate: 120 out of 640 questionnaires sent were returned, reply rate 19%. In contrast, the second lot had an acceptable reply rate: 54 out of 120 questionnaires sent were returned, reply rate 45%."

line 183-4,
Now this information has been included (lines 194 to 195 of the revised manuscript): "Veterinarians considered that a case of CanL was of local origin if dog lived at least during the transmission period in the area of their practice."

line 193.
It has already been detailed in lines 73 to 77 of the revised manuscript.

In addition, the question about the different languages was answered very superficially. Was this done in a formal way where the survey was then back translated and evaluated for meaning? I assumed it was designed by EDEN but can't find it on the website. Who developed it?
At several EDEN meetings all partners discussed questionnaire´s content. Finally, Jean Pierre Dedet and Clive Davies developed the questionnaire. Clive Davies sent it to our group in French and we translated it into Spanish.

As for the sensitivity and specificity of the tests used, is there commonly available testing that provides these where some estimate of accuracy could be obtained? Or are all different sources of tests usually used.
This matter is within "Results" heading, in lines (171-173): "In rank order, the diagnosis techniques mostly used were IFAT (91%), dipstick (29%), microscopy observation (26%), PCR (14%) and ELISA (11%)." In fact, IFAT is the serological technique most used in a routine diagnosis.