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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

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ABSTRACT

Leishmaniosis is present in the Mediterranean region of Europe, where *Leishmania infantum* is responsible for the disease, dogs are the main reservoir, and sand flies of the *Phlebotomus* genus, subgenus *Larroussius*, are proven vectors. Some areas, including Minorca in the Balearic Islands, are considered free of the disease, despite the presence of vectors. However, in the context of the current expansion of canine leishmaniosis in parts of Europe, an epidemiological study using a veterinary questionnaire was carried out to establish the current situation of the disease in the Balearic Islands. While 50% of veterinarians thought that the incidence of canine leishmaniosis had not changed over time, 26.2% perceived an increasing trend, mainly those from Minorca, where most of the veterinarians polled (88.1%) considered the new diagnosed cases as autochthonous. A cross-sectional serological study performed in this island gave a seroprevalence rate of 24%. Seroprevalence among animals of local origin and with no history of movements to endemic areas was 31%. The presence of autochthonous canine leishmaniosis in Minorca was not correlated with an increase in vector density. The environmental and climatic factors that influenced the distribution and density of *Phlebotomus perniciosus* on the island and the possible causes of the apparent emergence of canine leishmaniosis in Minorca are discussed.

1. Introduction

Leishmaniosis is present in the Mediterranean region of Europe, where *Leishmania infantum* is the causative agent of the disease (Alvar et al., 2004; Gállego, 2004). In the Balearic Islands, human (HL) and canine leishmaniosis (CanL) are considered to be highly endemic (Pujol et al., 2007; Riera et al., 2008).

Despite this, on the island of Minorca only one official case of HL has been recorded, corresponding to a cutaneous form in a 16-month-old child who had previously travelled to Ibiza (Boletín Epidemiológico Semanal, 1978–1994; Fulls setmanals de Vigilància Epidemiològica, 2002–2013). Three cases of HL in Minorca are recorded in the literature: one visceral case in a 23-year-old woman from Almeria (Spain), a cutaneous case in a young man from Valencia (Spain), another visceral case in a patient who had not left Minorca for 13 years, which was considered a possible reactivation of a cryptic leishmania infection acquired in Catalonia (Portús et al., 1994). Additionally, it is recorded a suspected case of infection detected through serological test in a blood donor during an epidemiological study carried out on the island without any history of travelling abroad (Riera et al., 2008).

Dogs are the domestic reservoir for *L. infantum* (Ashford and Bettini, 1987; Alvar et al., 2004; Gállego, 2004; Maroli et al., 2012). Despite the recommendations of the World Organization for Animal Health (OIE), which includes leishmaniosis in the list of notifiable terrestrial and aquatic animal diseases, there is no official data on the distribution and prevalence of CanL in Spain. The few published records of CanL in the Balearic Islands give rates of infection ranging from 0.9% to 77%, according to the island, the characteristics of the sample, and the diagnostic technique used

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(Matas-Mir and Rovira-Alos, 1989; Seguí, 1991; Portús et al., 1994; Solano-Gallego et al., 2001; Pujol et al., 2007; Cabezón et al., 2010). Unlike the other islands, the seroprevalence of CanL in Minorca is very low (0.9%), and the few published studies consider the disease as non autochthonous, since the few dogs found to be seropositive were all from endemic areas in Spain (Seguí, 1991; Portús et al., 1994).

Nevertheless, entomological studies have shown that the main proven vector of leishmaniosis in Spain, *Phlebotomus perniciosus*, is present in all the Balearic Islands, including Minorca (Gil Collado et al., 1989; Seguí, 1991; Gállego Berenguer et al., 1992; Portús et al., 1994).

Several factors have been considered as responsible for the emergence or reemergence of leishmaniosis, including globalization, climatic changes, increasing vector density, and the introduction of parasitized dogs from endemic areas (Seguí, 1991; Portús et al., 1994; Gállego, 2004; Ready, 2010; Gramiccia, 2011; Hartemink et al., 2011; Maroli et al., 2012). Emergence has been demonstrated in several foci in Europe (Dereure et al., 2009; Martín-Sánchez et al., 2009; Morosetti et al., 2009; Gálvez et al., 2010b; Branco et al., 2013), while in others it could not be proved due to an absence of preliminary data or the unknown origin of dogs (Miró et al., 2012; Ballart et al., 2013).

With the aim of investigating the current situation of CanL in the Balearic Islands using a standardized methodology, we distributed the EDEN (Emerging Diseases in a changing European eNvironment) veterinary questionnaire to veterinarian pet clinics. The results obtained from Minorca then prompted us to carry out an entomological and cross-sectional study on CanL seroprevalence in this island.

2. Materials and methods

2.1. Study area

The Balearic Islands are an archipelago located in the western Mediterranean region comprising four main islands (Majorca, Minorca, Ibiza and Formentera) and several smaller islands and islets. The geological origin of the Balearic Islands, with the exception of Minorca, is understood as a continuation of the Baetic Mountains, while Minorca is a continuation of the Pyrenees (Martín-Algarra and Vera, 2004). Apart from the high mountains of Majorca, the Balearic Islands have a Mediterranean climate.

In 2011, the Balearic Islands had a population of 1,093,568 inhabitants, with the majority living on Majorca (862,425 inhabitants), followed by Ibiza (129,562), Minorca (92,434) and Formentera (9147) (http://www.ibestat.cat/ibestat/page?lang=ca). The canine census in 2012 was of 250,596 dogs, with 13,956 on the island of Minorca (data provided by the *Col legi Oficial de Veterinaris de les Illes Balears*).

2.2. Canine leishmaniosis

2.2.1. Veterinary questionnaire survey

In 2009, to investigate the level of CanL awareness among local veterinarians in the Balearic Islands, a standard questionnaire employed in the EDEN Project (Gálvez et al., 2010b; Farkas et al., 2011; Ballart et al., 2013) was translated into the official local languages (Spanish and Catalan) and sent out by mail with pre-stamped envelopes to facilitate return. A conference was previously organized with the help of the *Col.legi Oficial de Veterinaris de les Illes Balears* to encourage veterinarians with pet clinics to become involved in the study. A total of 111 veterinary clinics were consulted. The questionnaire included questions about the kind of clientele, number of suspected and confirmed cases of CanL, symptoms and frequency with which they were observed, method of diagnosis, and the veterinarians' perception of the disease, including trends in CanL prevalence and control measures used (Gálvez et al., 2010b). A telephone call was made when no answer to the questionnaire was received. The questionnaire responses were transferred into a database in excel format and the locations of veterinary clinics were geocoded using Google Earth.

2.2.2. Cross-sectional study of canine leishmaniosis

A cross-sectional study on CanL was performed in April–June 2010 on 121 dogs from the island of Minorca by serological methods. The animals were selected, with permission from the owners, by the practitioners of three veterinary clinics located in different areas of Minorca (Mercadal, Ciutadella and Sant Lluis) (Fig. 1). To detect autochthonous cases of naturally acquired CanL, veterinarians were asked to select animals born on the island and without any history of travelling abroad regardless of the presence of clinical signs.

Blood samples, obtained by cephalic vein puncture, were collected in 5 ml tubes and sera obtained by centrifugation was frozen and conserved at −40 °C until use.

2.2.3. Serological analysis

As in other studies, we used four serological techniques: an in-house Immunofluorescent Antibody Test (IFAT), an in-house Enzyme-Linked Immunosorbent Assay (ELISA), an in-house Western Blot (WB) technique, and a commercial Immunochromatographic Test (ICF) (Ballart et al., 2013). Dogs that tested clearly positive with at least two immunological methods were considered seropositive and probably infected (Iniesta et al., 2002; Ballart et al., 2013). Dogs that tested positive with at least two techniques but were borderline were considered doubtful.

2.2.3.1. Speed Duo Leish K. A rapid and feasible commercial immunochromatographic dipstick test (ICF) for use in the field to detect anti-*Leishmania* kinesin antibodies (Speed® Leish K, BVT Group, Virbac) was used. The test was carried out on the serum following the manufacturer's instructions.

2.2.3.2. In-house Enzyme-Linked Immunosorbent Assay. An in-house Enzyme-Linked Immunosorbent Assay (ELISA) previously proven to be useful in epidemiological studies was performed as previously described, with some modifications (Riera et al., 1999). Briefly, sonicated promastigotes of a *Leishmania infantum* strain (MHOM/FR/LEM75/MON1) were used at a protein concentration of 20 μg/ml in 0.05 M carbonate buffer at pH 9.6. Sera were diluted to 1:400 in phosphate buffered saline-tween 1% milk (Sigma, St. Louis, MO, USA) and Protein A peroxidase (Sigma) (1:30,000) was used instead of the second antibody. The reaction was stopped with H_2SO_4 3 M and the optical densities were measured at 492 nm using a Titertek Multiskan PlusMKII (Flow Laboratories International, SA, Lugano, Switzerland). The reaction was quantified in units (U) by reference to a positive serum arbitrarily set at 100 U. The cut-off was established at 24 U (Riera et al., 1999).

2.2.3.3. Western Blotting. An in-house Western Blotting (WB) technique previously proven to be useful and highly sensitive in epidemiological studies was performed as described elsewhere, with some modifications (Riera et al., 1999). A concentration of 3×10^8 promastigotes/ml (*L. infantum* (MHOM/FR/LEM75/MON1)) in sample buffer (0.5; Tris–HCl, ph 6.8, 0.01 M EDTA, 5% SDS, 5% 2-mercaptoethanol, 0.0125 bromophenol blue) boiled for 5 min was used as the antigen. Electrophoresis on a Mini-gel AE 6400 Dual Mini Slab Kit (Atto, Bunkyo-Ku, Japan) was performed on