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12. Is leishmaniosis spreading to northern areas of the Iberian Peninsula? The examples of Lleida (NE Spain) and Andorra

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Abstract. The entomological and canine leishmaniosis surveys carried out in the northwest of Catalonia and in Andorra in the context of the European project Emerging Diseases in a changing European eNvironment (EDEN) are summarized. The aim of the study was to obtain data on the presence of leishmaniosis in these areas and the spatial distribution of their vectors.

Introduction

Global changes in climate, human activities and migration have resulted in the emergence or re-emergence of vector-borne diseases, such as leishmaniosis, in some parts of the world [1-5]. Leishmaniosis is a transmissible disease that affects man and other mammals and is caused by different species of the genus *Leishmania* [6]. In Europe it is caused by *Leishmania infantum* and dogs are the principal reservoirs [2].

The highly specialized transmission of leishmaniosis is by the bite of hematophagous insects acting as vectors and is restricted to a group of dipterans,

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the phlebotomine sand flies [2,7], which are found mainly in warm parts of the world [8]. Two genera are involved in the transmission of leishmania parasites: *Phlebotomus* in the Old World and *Lutzomyia* in the New World. In Spain, two species of the genus *Phlebotomus*, subgenus *Larroussius*, *P. ariasi* and *P. perniciosus*, are the proven vectors, acting in sympatric conditions in some of the foci [3,9-11]. Studies carried out on phlebotomine richness in Spain identified 12 sand flies species, 10 of the *Phlebotomus* genus and 2 of *Sergentomyia*, each one with a different distribution in the Iberian Peninsula [12-17]. As in other countries, the focalized geographical distribution of sand flies in Spain has been related with climatic and environmental characteristics, but few in-depth studies exist on the influence of such variables on the distribution of sand flies in local areas [17-19].

Human leishmaniosis (HL) is present in 98 countries around the world [20]. In humans, the disease is usually manifested as visceral or tegumentary forms, cutaneous cases being more frequent [4]. In recent years, the cryptic form of HL has also been described, mostly among blood donors [21]. Until recently, in Europe HL was considered to be present mainly in the Mediterranean Basin, but it is now spreading and emerging in zones previously thought to be free, as well as increasing in endemic areas [22-26]. HL has been reported in northern and eastern countries of Europe, although some cases are considered imported [27]. In Spain, the first case of HL was described by Pittaluga in 1912 [28], after which many cases were diagnosed in the Iberian Peninsula. The disease began to decrease at the end of the 1940s, probably due to the reduction of vectors by insecticide use and also a decline in reservoirs after a civil war and a period of severe economic depression [11,29,30]. In the 1980s the disease increased considerably due to opportunistic infection in VIH patients [31]. From 1982 until 1996, HL was a notifiable disease in Spain, but its notification is currently mandatory in only 12 of the country's 17 autonomous communities, including Catalonia.

Canine leishmaniosis (CanL) is a widespread disease, present in 42 countries in Europe, Africa, Asia and America, and suspected in three other countries of Africa [2]. The disease is included in the list of the World Organization for Animal Health (OIE), being considered as important from the socio-economical and sanitary points of view. As dogs constitute the main reservoir hosts of the parasite, this represents a risk for humans [2,3], particularly considering the high number of asymptomatic animals [32-34]. In Western Europe, CanL is considered highly endemic in some parts of Portugal, Spain, France and Italy. In recent years, the disease has been increasing in classical areas [19,25,26] and has spread to northern regions considered non-classical for CanL [23,24,35]. However, cases in northern and eastern Europe have been detected in dogs that have travelled with their

owners to the Mediterranean basin or been bought in endemic areas [27]. CanL can manifest itself with a great variety of unspecific cutaneous and visceral symptoms, which hampers clinical diagnosis [36]. Cryptic leishmaniosis in dogs has been described [36,37]. The first case of CanL in Spain was reported by Pittaluga in 1913 [38]. Although nowadays the disease is very widespread in the country and well known by veterinarians, no official data exists on its prevalence, which seroepidemiological studies situate in a range of 1.6% in the North to 34.6% in the South [39,40]. The data on CanL distribution is also incomplete, with very little available for the north of the country.

The present work summarizes epidemiological studies on leishmaniosis carried out in Andorra and Spain (Catalonia, Lleida province) in the course of the European project Emerging Diseases in a changing European eNvironment (EDEN).

1. Andorra. Entomological survey

Andorra is a small European country of 468 km^2 located in the Pyrenees, consisting predominantly of rugged mountains, with the lowest point at 840 m a.s.l., in the southern basin of the Gran Valira, and the highest at the 2946 m Coma Pedrosa peak. It is characterized by a Mediterranean high mountain climate with cold temperatures in winter and mild summers, with considerable variations depending on altitude and orientation. The country is divided in 7 administrative parishes: Canillo, Encamp, Escaldes-Engordany, La Massana, Ordino, Sant Julià de Lòria and Andorra La Vella.

No data exist on leishmaniosis in Andorra, despite the disease being well known in its neighbouring countries of Spain and France [24,37,41]. The only existing report on the phlebotomine fauna in this country describes the survey of one collection site, where two sand fly species were captured, *S. minuta* and *P. ariasi* [15]. Since the presence of the vector is the focusing element for a transmissible disease and data on sand fly vectors in Andorra was lacking, we decided to carry out an entomological survey.

39 stations were sampled during July 2007 covering all the parishes except Andorra La Vella. Sticky traps (20 cm× 20 cm sheets of papers covered in castor oil) were used and set in adult sand fly resting places [7,42] for four days (Fig. 1). Because of the highly mountainous topography of the region, captures were made along transects [7], following all the main roads. The sand flies were fixed until their morphological identification following the keys of Gállego Berenguer et al. (1992) [13].

Figure 1. Sticky traps used in the capture of sand flies: Examples of a sampling site and adhesive paper with captured sand flies.

A total of 21 specimens belonging to two species of the genus *Phlebotomus*, *P. ariasi* (5 females, 13 males) and *P. perniciosus* (1 female, 2 males) were captured in 10 sampling sites [43]. Global results of number, density, abundance and frequency are summarized in Fig. 2. The characteristics of vector density according to altitudinal distribution are summarized in Fig. 3.

The geographical distribution of the vectors and their densities are represented in Fig. 4. *P. perniciosus* is restricted to the south of the country, in Sant Julià de Lòria parish, at altitudes below 1,000 m a.s.l., whilst *P. Ariasi* covers a wide area, from 800 m to 2,200 m a.s.l., being present in all the 6 parishes where traps were placed [43]. The results obtained in Andorra are in

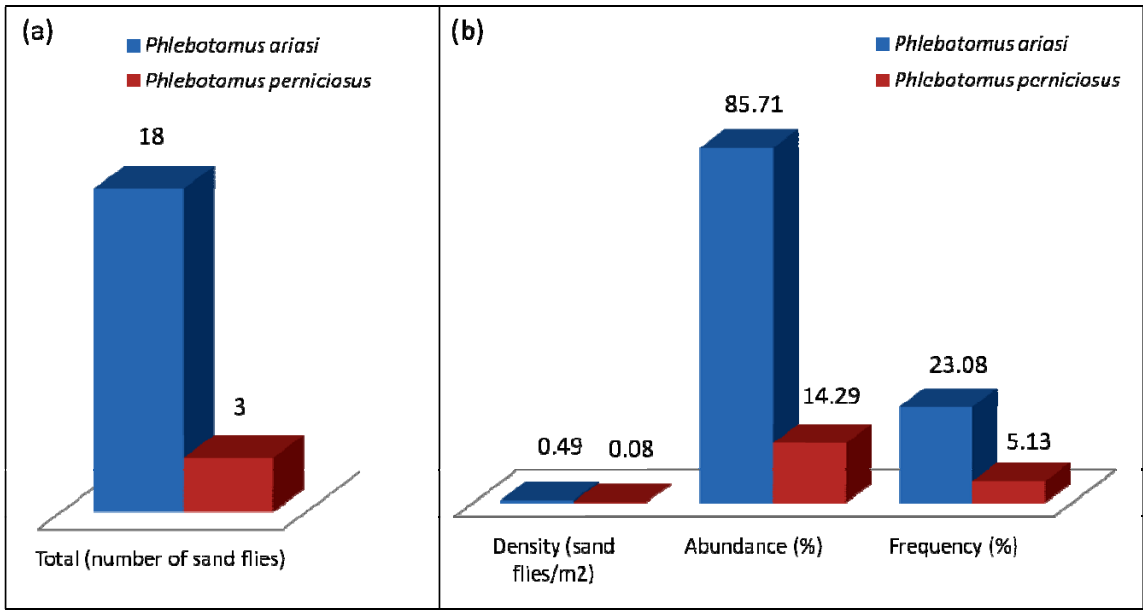

Figure 2. (a) Number, (b) density (number of sand flies per square metre of sticky trap), abundance (relative number of captured sand flies of one species related to the total number of sand flies captured and expressed as a percentage) and frequency (relative number of positive stations for one species expressed as a percentage) of *P. ariasi* and *P. perniciosus* in Andorra.

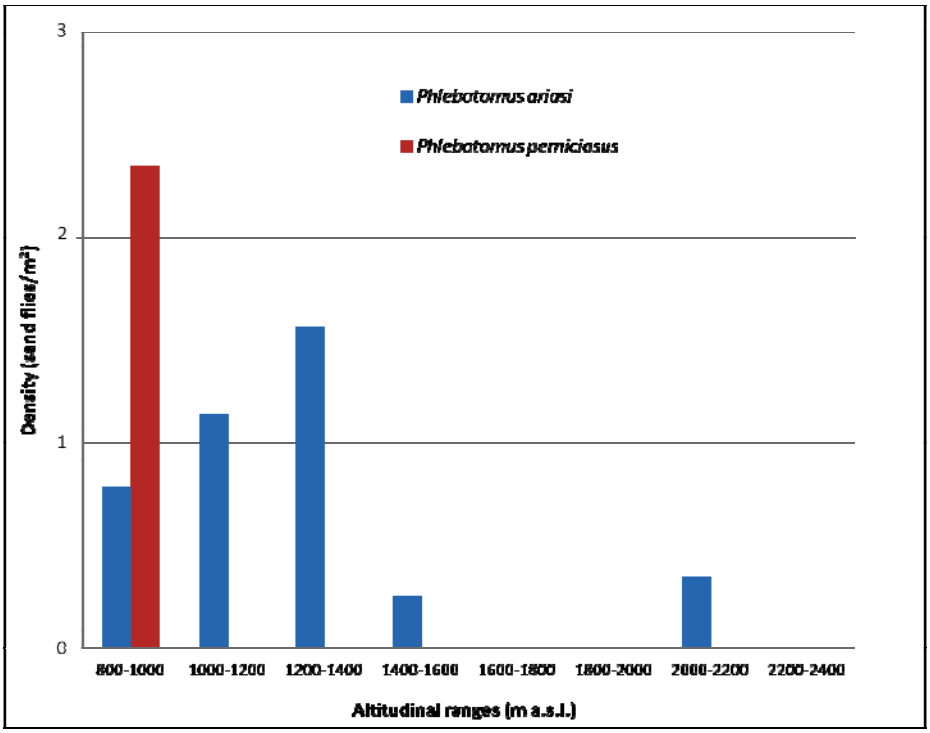

Figure 3. Density (number of sand flies per square metre of sticky trap) of *P. perniciosus* and *P. ariasi* at different altitudinal ranges in Andorra.

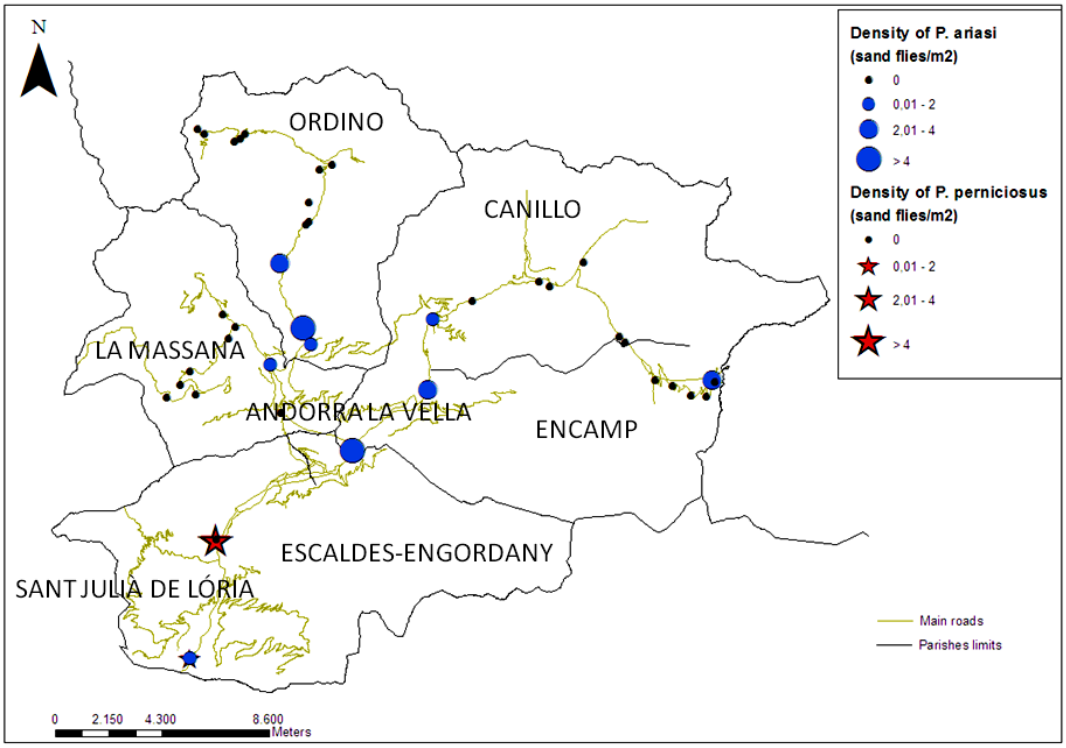

Figure 4. Sampling sites in Andorra showing the presence and density ranges of *P. ariasi* and *P. perniciosus*.

agreement with the predominant presence of *P. ariasi* in humid or sub-humid areas with cold winters, being the only potential vector of leishmaniosis in cold zones, and the preference of *P. perniciosus* for semi-arid or subhumid zones with warm winters and mild summers [44,45].

This entomological survey resulted in the first reporting of *P. perniciosus* in Andorra, and gave new insight into the geographical and altitudinal distribution of *P. ariasi* in this country. *P. ariasi* was captured at altitudes above 2,000 m a.s.l., which is unusual in the Mediterranean area [46].

Vector presence is considered to be a risk factor for the emergence of leishmaniosis in temperate Europe [3]. As both species are responsible for the transmission of *L. infantum* in bordering France and Spain [24,44], we therefore consider Andorra to be an area at risk for the emergence of the disease. The arrival of infected dogs from endemic areas, without surveillance and control measures, could lead to the establishment of the disease in this country [43]. The summer temperature in Andorra would allow the development of the leishmania life cycle in the vector, as has been experimentally demonstrated for *P. ariasi* [7], and its transmission to the vertebrate hosts.

2. Lleida province

The province of Lleida is situated in the NE of Spain (Catalonia), bordering with the Pyrenean areas of Andorra and France in the North. It covers an extension of 12,173km^2 and occupies a great variety of habitats, including plains and mountains (Pyrenees). The altitude ranges from just over sea level to 3,143 m a.s.l. of the Pica d'Estats mountain. Climate is highly variable according to altitude, ranging from Mediterranean High Mountain in the mountainous areas in the north to a continental climate in the central depression.

Before our survey, only one partial entomological survey had been carried out in Lleida province [44], reporting a sand fly fauna of 5 species (*S. minuta*, *P. perniciosus*, *P. ariasi*, *P. sergenti* and *P. papatasi*), including the vectors of *L. infantum*. Some HL cases had been noted in the epidemiological bulletins [47], but no data on CanL was available.

Due to the limited knowledge of sand fly fauna in Lleida, we decided to carry out an entomological study throughout the province. The EDEN methodology was applied so the results would be comparable with those obtained by our survey in Andorra and by other European teams participating in the project. Another aim was to obtain HL and CanL data in the province for the first time.

2.1. Entomological survey

A cross-sectional study was carried out in July 2006 in Lleida province. Sand flies were captured using sticky traps, as in Andorra. A total of 4100 sticky traps were placed in all the 13 counties of the province. Due to the large extension and physical characteristics of the region, captures were made by transects following the main roads, which enabled the whole territory to be covered [7].

Five species of sand flies were captured, the densities shown in Table 1. All were previously identified in the province [44]. The results obtained show that the two vector species of *L. infantum* are present in Lleida province. Neither species was found in Pla d'Urgell county. *P. ariasi* was not present in the southern counties (Garrigues and Segrià), probably because this species mainly inhabits humid or sub-humid zones with cold winters, and *P. perniciosus* was not found in Vall d'Aran, which is the coldest and wettest county.

Table 1. Density (number of sand fly species captured per square metre of sticky trap).

Density (sandflies/m^2 sticky trap)					
County	*P. ariasi*	*P. perniciosus*	*P. sergenti*	*P. papatasi*	*S. minuta*
Alt Urgell	1.47	2.76	0	0	33.41
Alta Ribagorça	2.74	3.48	0	0	13.37
Garrigues	0	6.56	0	2.38	31.62
La Cerdanya	0.31	1.25	0	0	0.94
Noguera	0.49	12.86	0.22	0.45	33.04
Pallars Jussà	1.78	4.83	0.77	0.05	76.14
Pallars Sobirà	1.70	1.51	0	0	54.91
Pla d'Urgell	0	0	0	0	77.68
Segarra	0.06	7.84	0.48	3.13	22.96
Segrià	0	0.91	0	0.86	16.77
Solsonès	2.09	1.83	0	0.04	1.44
Urgell	0.03	7.85	0.07	1.29	22.33
Vall d'Aran	0.98	0	0	0	0

Global results on the number, density, abundance and frequency of leishmania vectors are summarized in Fig. 5. The characteristics of vector density according to altitudinal distribution are summarized in Fig. 6 and their geographical distribution among the counties of Lleida is included in Fig 7.

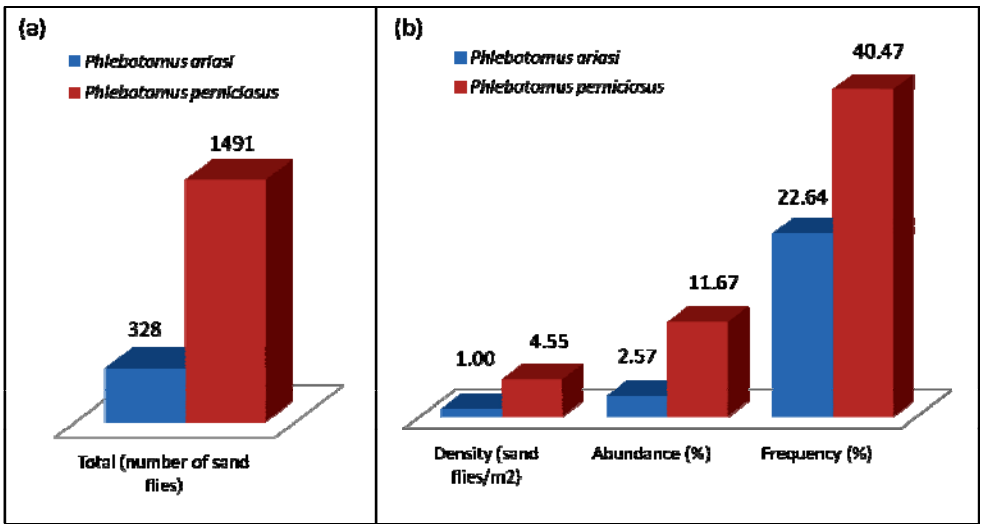

Figure 5. (a) Number, (b) density (number of sand flies per square metre of sticky trap), abundance (relative number of sand flies of one specie captured related to the total number of sand flies captured and expressed as a percentage) and frequency (relative number of positive stations for one specie expressed as a percentage) of *P. ariasi* and *P. perniciosus* in Lleida province.

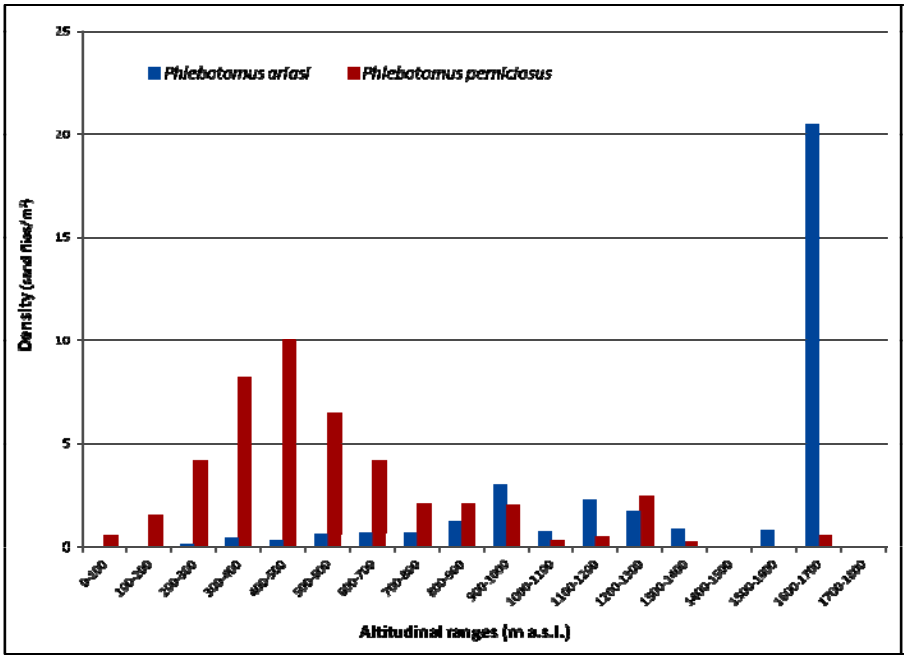

Figure 6. Density (number of sand flies per square metre of sticky trap) of *P. perniciosus* and *P. ariasi* at different altitudinal ranges in Lleida province.

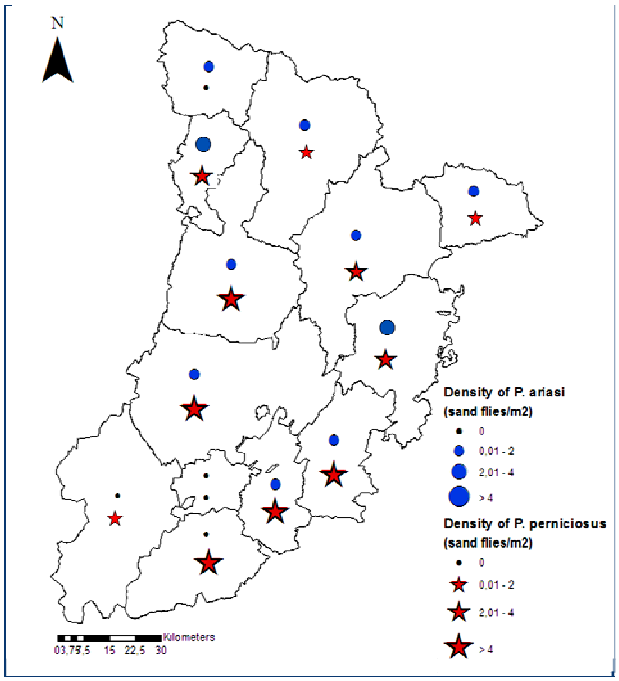

Figure 7. Global density (number of sand flies per square metre of sticky trap) of the two vector species in the different counties of Lleida province.

The density of P. perniciosus was highest at altitudes below 800m a.s.l, while the density of P. ariasi was highest at altitudes above 800m a.s.l. Both vector species have a wide distribution, but *P. perniciosus* was captured principally in southern counties and *P. ariasi* in northern ones, suggesting that each species can potentially act as the principal vector, depending on the area.

2.2. Human leishmaniosis

From 1982 to 2012, 900 HL cases were officially recorded in Catalonia [47], 39 of them in Lleida province (Fig. 8). The official data do not include the origin of the patients nor the sanitary centres where the diagnoses were made. Moreover, no data is included regarding the patients' movements within Spain and travel history to other endemic countries. The only published case of HL in the province of Lleida was diagnosed in a hospital far from the Pyrenean area and without any data of travels to other endemic regions [48]. So the presence of sporadic cases of HL is not conclusive proof of the existence of an autochthonous focus.

2.3. Canine leishmaniosis

A questionnaire on CanL, designed by the EDEN project to rapidly obtain information from veterinarians about the presence, diagnosis and

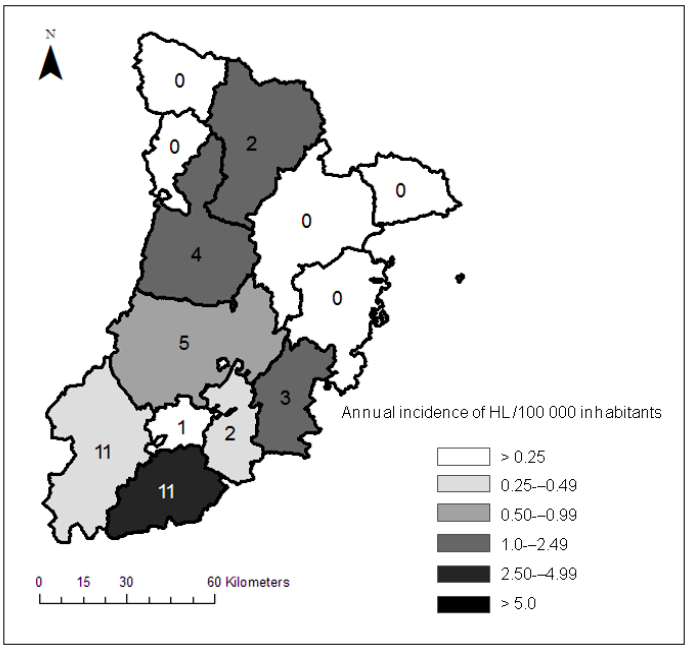

Figure 8. Distribution of human leishmaniosis in Lleida. Human data recorded from the *Butlletí Epidemiològic de Catalunya* (1982–2012) (annual incidence rate was calculated from the number of cases declared during 1982–2012 and the census of 1996). The numbers included in the different counties represent the number of human leishmaniosis cases during 1982–2012 (2012 data only for weeks 1 to 12) (updated from [49]).

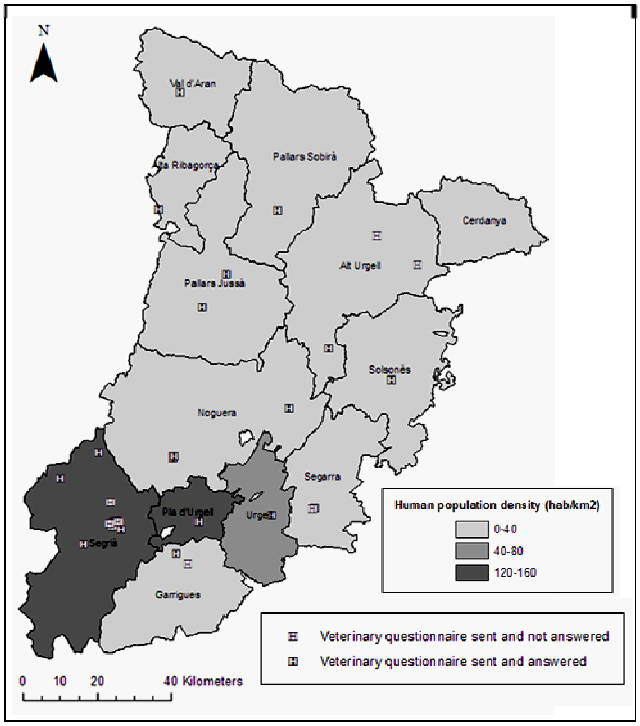

Figure 9. Study area (Lleida province and Pallars Sobirà county), surveyed points for CanL and veterinarian questionnaires [50].

perception of the disease, was applied throughout the province of Lleida (Catalonia, NE of Spain) in 2009. A total of 41 questionnaires were sent to all the veterinarians with a pet veterinary clinic (Fig. 9).

Despite the small number of questionnaires sent out, the response (78%) was considerably high, which could be related with the extent of veterinarian awareness of the disease and the widespread opinion that canine leishmaniosis has increased among patients during the last ten years (78.1%). In our study, the dogs attending the veterinarian clinics were mainly from mixed (rural and urban) areas (71.9%), reflecting that Lleida is a region composed of towns and villages with agricultural and farming activities [50]. A high percentage of the veterinarians thought CanL cases were new and acquired locally (93.8%), which plays in favour of an active focus in the region. All the practitioners (100%) recommended actively preventive measures, particularly collars and spot-on products, as in other European regions [51-53]. This could be related with the general veterinary opinion that local CanL cases had increased (78.1%) and that the infections were acquired in the area (93.8%), leading them to consider the disease as a real epidemiological problem [50].

Additionally, a cross-sectional serological study of CanL was carried out in October 2009 in Pallars Sobirà, a county of the province of Lleida located in the Spanish Pyrenees. Blood samples of 145 dogs were obtained by cephalic vein puncture (Fig. 10). Owner data, dog characteristics, geographical coordinates and altitude data were collected.

Dog samples were analyzed by four serological techniques: 1) an in-house immunofluorescent antibody test (IFAT), 2) an in-house enzyme-linked immunosorbent assay (ELISA), 3) an in-house Western Blot (WB) technique, and 4) a commercial immunochromatographic test (ICF) for the detection of circulating anti-*Leishmania* kinesin antibodies. Dogs that tested positive with at least two immunological methods were considered seropositive and probably infected [54].

Figure 10. Blood samples were obtained by cephalic vein puncture.

The long tradition of hunting among the inhabitants of Pallars Sobirà explains why a high percentage of dogs in the study were hunting dogs living in kennels in a rural environment (54%). Dogs of this kind of population have been considered as sentinel indicators of diseases of veterinary and zoonotic interest [55]. Seropositivity results are shown in Fig. 11.

The seroprevalence reported in this study (33.1%), in an area where the presence of the disease was unknown, is high considering that the highest seroprevalence value found in a known focus in Spain is 34.6% [39]. Nevertheless, this figure may be an overestimation, due to the biased nature of the sample. The analyzed dogs lived mainly outdoors in optimal conditions for disease transmission [25,26,37,56,57], due to the predominant exophilic and exophagic character of most sand fly species taking a blood meal at twilight or night [8]. The density of dogs living in kennels is another factor that facilitates parasite transmission, both directly and by vector, as previously mentioned and reviewed [36,58]. The finding of a high percentage of seropositive asymptomatic dogs (70%) prompts us to hypothesize that the emergence of CanL in Pallars Sobirà county is not very recent [50]. This idea is supported by a seroprevalence threshold usually associated with steadily established CanL foci (2.5%) [59].

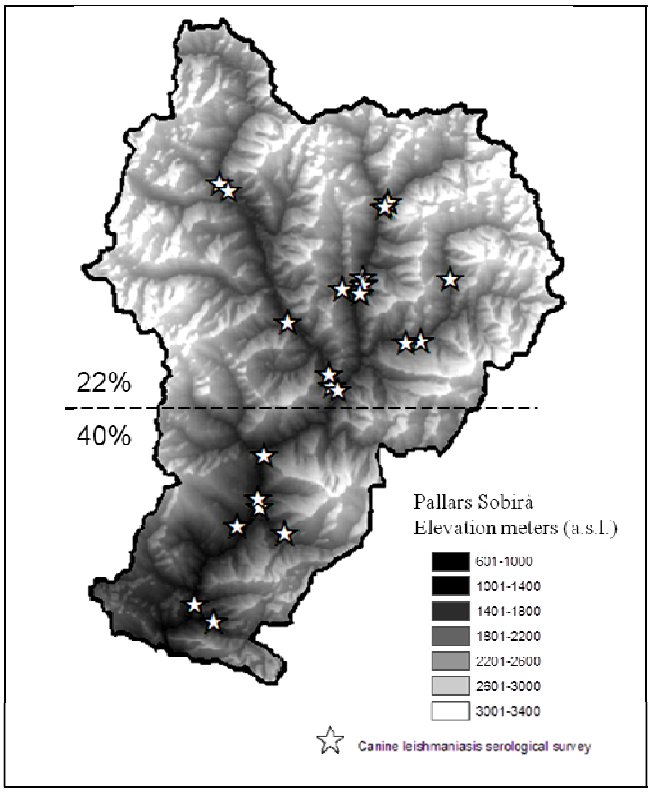

Figure 11. CanL serological survey and seroprevalence found in the north and south of Pallars Sobirà county [50].

Despite the biased results, our study of kennel dogs of local origin proved useful for detecting an autochthonous focus of leishmaniosis through the analysis of a small number of animals (145 dogs) [50].

3. Conclusion

The data on HL, obtained from the Epidemiological Bulletins, and on CanL, from the questionnaire completed by local veterinarians, suggest that both types of the disease are present in Lleida province. Nevertheless, the lack of information on the origin and travel histories of the human cases published in the Epidemiological Bulletins undermines their validity as markers of leishmaniosis distribution. The cross-sectional serological study carried out in Pallars Sobirà county allowed the detection of an autochthonous focus of CanL, which is suspected not to be of recent emergence, and confirms the presence of CanL in Lleida province. Since dogs, particularly those from rural areas, travel less than humans, CanL is a more accurate marker of the real distribution of leishmaniosis in a given territory.

The entomological surveys carried out in Andorra and Lleida confirm the presence of the two proven vectors of *L. infantum*, *P. ariasi* and *P. perniciosus*, suggesting that these territories are at risk of leishmaniosis transmission. More accurate data on the real distribution and density of the vectors are required. Studies on factors that could influence vector distribution, including climatic and environmental factors, are also necessary. The surveillance of dogs arriving from other areas where leishmaniosis is endemic is advisable to avoid or control the possible establishment of the disease in Andorra. Further serological studies on a more representative dog population of Lleida are required to know the true extent and prevalence of the infection.

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