Leishmania infantum in free-ranging hares, Spain, 2004-2010

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Iberian hares (*Lepus granatensis*) were recently deemed responsible for an outbreak of human leishmaniasis affecting metropolitan Madrid, Spain. However, the reservoir potential of hares in Europe is poorly known. We report a retrospective survey on *Leishmania infantum*, the causal agent of zoonotic endemic leishmaniasis in the Mediterranean basin, infection status of Iberian, European (*Le. europaeus*) and Broom (*Le. castroviejoi*) hares in Spain. Spleen samples from 94 hares were tested by polymerase chain reaction. Sequencing and restriction fragment length polymorphism (RFLP) assays were performed on positive samples and RFLP patterns compared with those of strains reported in the scientific literature. DNA prevalence in hare spleen samples was 43.6% (95% confidence interval: 33.6-53.6). In all six regions studied at least one positive sample was found. RFLP revealed existence of specific hare strains of *L. infantum* differing from those reported in wild carnivores in Spain. The widespread presence of *L. infantum* in the most abundant Spanish hare species and the recent evidence of the ability of naturally infected hares to transmit the pathogen to *Phlebotomus perniciosus*, its main vector in the western Mediterranean, suggest that hares may have an unexpected role in the epidemiology of *L. infantum* in Spain.

Introduction

Diseases at the wildlife-livestock-human interface are an increasing concern for public health, animal health and animal conservation authorities worldwide [1]. Also, wildlife-associated infectious diseases are at the top of human emerging diseases [2]. Basic epidemiologic knowledge would constitute the foundation for targeted prevention and control measures of wildlife-associated diseases, but knowledge is scarce for many of the currently emerging threats; Leishmaniasis in Europe is a good example. Endemic Mediterranean leishmaniasis is a disease of animals and humans caused by *Leishmania infantum*, a protozoan causing both visceral and cutaneous zoonotic leishmaniasis in the Mediterranean basin. *L. infantum* has recently spread northward from Mediterranean to temperate climates in Europe (e.g. Hungary and northern Italy), apparently linked to climate change [3] but perhaps also linked to increased movements of infected hosts, mostly dogs, from endemic Mediterranean areas [4]. Thus, leishmaniasis caused by *L. infantum* can be considered as a potentially emerging threat for central and northern European countries [4].

Dogs are deemed as major reservoirs of *L. infantum* since they efficiently replicate the protozoan parasite and are preferred hosts for vector phlebotomine sandflies [4]. Wild carnivores such as the wolf (*Canis lupus*), the red fox (*Vulpes vulpes*), the Egyptian mongoose (*Herpestes ichneumon*), the genet (*Geneta geneta*), the pine marten (*Martes martes*) or the Iberian lynx (*Lynx pardinus*) have also been implicated in the maintenance of *L. infantum* [5,6]. Recently, naturally infected Iberian hares (*Lepus granatensis*) were found to efficiently allow infection of *Phlebotomus perniciosus* sandflies with *L. infantum* [7]. Iberian hares were deemed as the main reservoirs of a leishmaniasis outbreak causing more than 260 human cases in the southwestern metropolitan area of Madrid since 2009 [7,8].

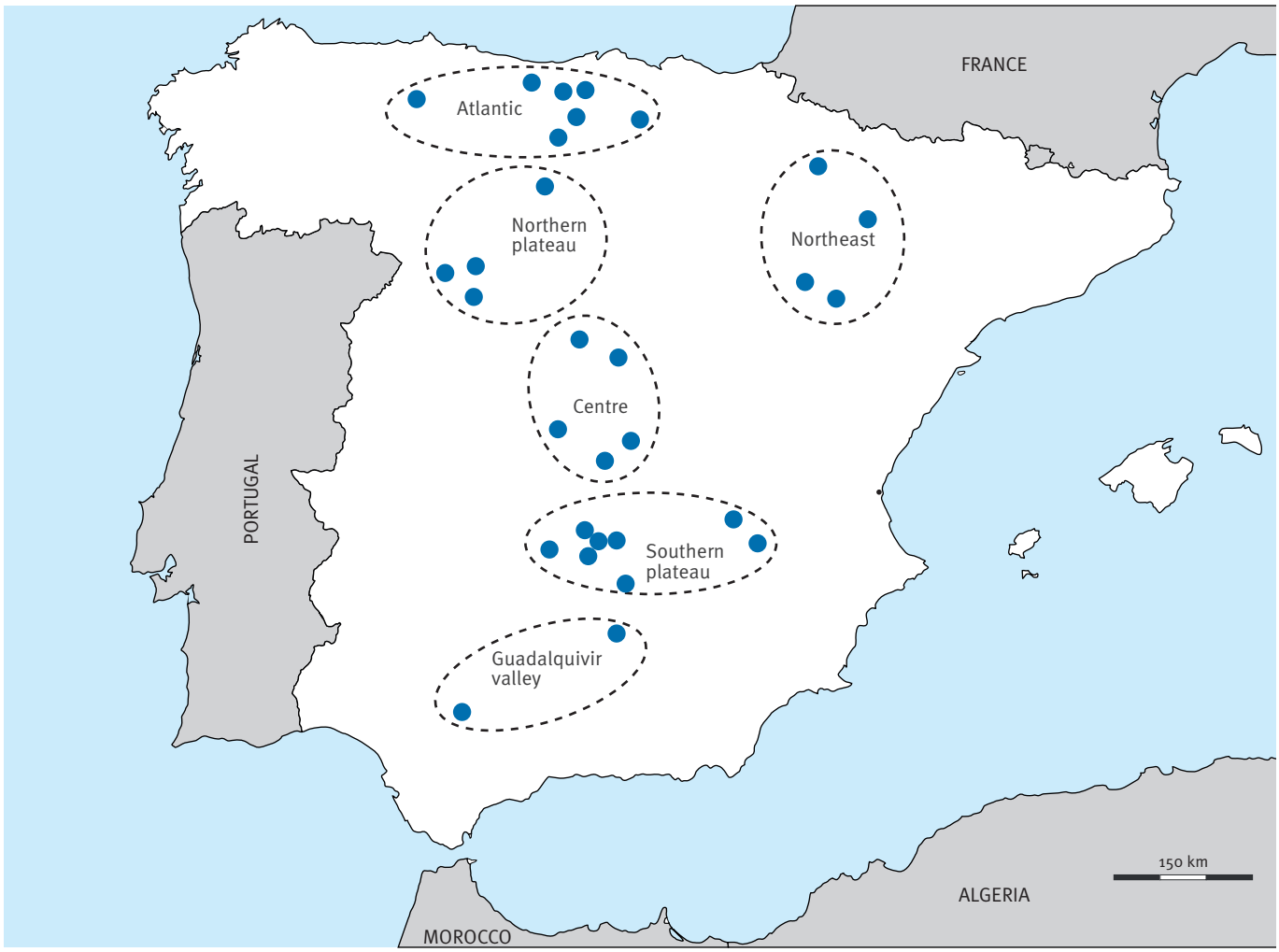
Three different hare species inhabit the Iberian Peninsula: (i) the Iberian hare that is present in vast areas of the Iberian Peninsula, (ii) the European brown hare (*Le. europaeus*) that lives in northern and northeastern Spain, and (iii) the Broom hare (*Le. castroviejoi*) that can be found in the Cantabrian Mountains [9]. Since basic information on the relationship between *L. infantum* and Iberian hare species is only anecdotal [7] and *Le. granatensis* populations are abundant and stable [10, unpublished data], we designed a retrospective survey on samples collected from Spanish hares through wildlife disease surveillance programs.

Methods

Spleen samples from a subset of hares (n=94) collected during necropsies performed over carcasses of animals found dead or harvested by hunters in Spain from 2004 to 2010 were used in this study. Sample collection was opportunistic since they were obtained

Figure

Location of hare sampling points by geographic region, Spain, 2004-2010



through Spanish wildlife disease surveillance programs. Hare tissues were preserved frozen at -20ºC until analysed.

According to their origin, samples were allocated to six geographic regions (listed here from north to south): Atlantic, Northern plateau, Northeast, Centre, Southern plateau and Guadalquivir river valley (Figure).

Total genomic DNA from spleen samples was extracted using a commercial kit (GenomeElute, Sigma-Aldrich, St. Louis, MO) following the manufacturer's protocol. A previously described polymerase chain reaction (PCR) targeting a 145 bp fragment present on the high copy of kDNA minicircles of *L. infantum* was performed on spleen samples [11]. PCR products were elicited by PCR fragment size estimation in comparison with two molecular weight standards: PCR 100 pb Low Ladder and pBR 322 HaeIII Digest (Sigma–Aldrich, St. Louis, MO) after electrophoresis in 2% agarose gel. Amplicons from 23 positive hares were sequenced to confirm *L. infantum* infection. Moreover, a restriction fragment length polymorphism (RFLP) assay was performed on all positive samples to compare amplicon patterns with previous studies from peninsular Spain [5]. For the RFLP assay, 15 µl of PCR product were digested with restriction enzymes BsiY I and Mlun NI as previously reported [12].

Basic comparison of prevalence values between sexes, species and geographic regions was performed by means of chi-squared tests. Statistical uncertainty was assessed by calculating the 95% confidence interval (CI) for each of the proportions according to the expression 95% CI. = $1.96[p(1 - p)/n]^{1/2}$ (where "p" is the proportion in its unitary value and "n" is the sample size) and expressed in percentage.

Table 1

Prevalence of *Leishmania infantum* infection in hares by geographic region and species, Spain, 2004-2010 (n=94)

Geographic region	Hare species	Number of samples	Positive	Prevalence in percent (95% CI)
Atlantic	*Le. europaeus*	14	9	64.3 (39.2-89.4)
	Le. castroviejoi	2	0	0.0 (n.a.)
Northern plateau	*Le. granatensis*	5	1	20.0 (0.0-55.1)
Northeast	*Le. europaeus*	2	0	0.0 (n.a.)
	Le. granatensis	5	3	60.0 (17.1-100.0)
Centre	*Le. granatensis*	10	6	60.0 (29.6-90.3)
Southern plateau	*Le. granatensis*	54	21	38.8 (21.8-51.8)
Guadalquivir river valley	*Le. granatensis*	2	1	50.0 (0.0-100.0)
Total		**94**	**41**	**43.6 (33.6-53.6)**

CI: confidence interval; Le: *Lepus*; n.a.: not applicable.

IBM SPSS 19.0 Statistical Package software (IBM Corporation, New York, USA) was employed for statistical analyses.

Results

Spleen samples analysed belonged to *Le. granatensis* (n=76; 24 males, 29 females and 23 unsexed), *Le. europaeus* (n=16; 5 males, 8 females and 3 unsexed) and *Le. castroviejoi* (n=2; both females).

The collected hare species were from the (i) Atlantic region:14 *Le. europaeus* and two *Le. castroviejoi* ; (ii) Northern plateau region: five *Le. granatensis*; (iii) the Northeastern region: five *Le. granatensis* and two *Le. europaeus*; (iv) Centre region: 10 *Le. granatensis*; (v) Southern plateau region: 54 *Le. granatensis*; and (vi) Guadalquivir river valley region: two *Le. granatensis*.

Overall, 41 out of 94 Spanish hares (43.6%; 95% CI: 33.6 to 53.6) were positive for the presence of *L. infantum* DNA. At least one positive hare was found in each of the six geographic regions surveyed (Table 1). Both Iberian and European hares tested positive for presence of *L. infantum* DNA by PCR.

No statistically significant differences in prevalence were observed between sexes: males (n=29) 44.8% (95% CI: 26.8 to 62.8) and females (n=39) 46.2% (95% CI: 30.2 to 62.2); species: *Le. granatensis* 42.1% (95% CI: 31.1 to 53.1), *Le. europaeus* 56.3% (95% CI: 32.3 to 80.3) and *Le. castroviejoi* 0%; and regions (Table 1). Interestingly, the highest prevalence value was observed in hares from Central and Atlantic regions.

Sequencing was successful from nine hares and homology with *L. infantum* kinetoplast DNA ranged from 94% to 99%.

Twenty-two RFLP patterns were obtained from 32 hares (see Table 2). Thirteen RFLP patterns were found in thirteen individuals; six from the Atlantic region, six from Southern plateau region and one from Centre region. Eight patterns were each present in two different hares; two of the patterns were exclusively present in hares from Southern plateau region and one was only present in hares from Centre region, while five of these eight patterns were present in hares from different geographic regions – two in hares from Atlantic and Southern plateau regions respectively, one in hares from Northern plateau and Southern plateau regions, one in hares from Centre and Southern plateau regions and one in hares from Atlantic and Centre regions. Finally, one of the 22 patterns was present in three different hares: two from Southern plateau and one from Northeast region.

Nine different patterns were found in the nine positive hares (all *Le. europaeus*) from Atlantic region (Table 2). Five hares from Centre region (all *Le. granatensis*) presented four different patterns. Sixteen hares from Southern plateau (all *Le. granatensis*) presented 13 different patterns. RFPL patterns identified in one hare from Northeast region and one hare from Northern plateau were also present in other regions.

No similarities were found between hare RFLP patterns and those previously found in wild carnivores from continental Spain [5].

Discussion

This study shows that *L. infantum* is present in two of the three Spanish hare species and that specific 'hare strains' of *L. infantum* circulate in Spain. However, the low number of samples from *Le. castroviejoi* – an endangered species – prevented determining if they are exposed to *L. infantum*. We selected testing the presence of *L. infantum* DNA in spleen samples instead of detecting antibody presence because we aimed to measure the occurrence of effective infections rather than detecting exposure. The effect of possible local

Table 2

Allocation to geographic region of the 22 *Leishmania infantum* restriction fragment length polymorphism patterns identified from hares, Spain 2004-2010 (n=32)

Hare number	RFLP pattern number	Species	Geographic region
1	1	*Le. europaeus*	Atlantic
2	2	*Le. europaeus*	Atlantic
3	3	*Le. granatensis*	Southern plateau
4	4	*Le. granatensis*	Southern plateau
5	4	*Le. granatensis*	Southern plateau
6	5	*Le. europaeus*	Atlantic
7	6	*Le. europaeus*	Atlantic
8	6	*Le. granatensis*	Southern plateau
9	7	*Le. europaeus*	Atlantic
10	8	*Le. granatensis*	Northern plateau
11	8	*Le. granatensis*	Southern plateau
12	9	*Le. granatensis*	Southern plateau
13	10	*Le. granatensis*	Centre
14	11	*Le. granatensis*	Southern plateau
15	12	*Le. europaeus*	Atlantic
16	13	*Le. granatensis*	Southern plateau
17	13	*Le. granatensis*	Centre
18	14	*Le. europaeus*	Atlantic
19	14	*Le. granatensis*	Centre
20	15	*Le. granatensis*	Southern plateau
21	15	*Le. granatensis*	Southern plateau
22	16	*Le. granatensis*	Southern plateau
23	17	*Le. granatensis*	Southern plateau
24	18	*Le. granatensis*	Centre
25	18	*Le. granatensis*	Centre
26	19	*Le. granatensis*	Southern plateau
27	19	*Le. granatensis*	Northeast
28	19	*Le. granatensis*	Southern plateau
29	20	*Le. granatensis*	Southern plateau
30	21	*Le. granatensis*	Southern plateau
31	21	*Le. europaeus*	Atlantic
32	22	*Le. europaeus*	Atlantic

Le: *Lepus*; RFLP: restriction fragment length polymorphism.

temporal trends in *L. infantum* prevalence caused by changes in vector and host population dynamics could have had an effect on prevalence rates found in this study.

In spite of sample size limitations in this study, overall *L. infantum* DNA prevalence in *Le. granatensis* and *Le. europaeus* seems to be above the 30% (lower limits of estimated confidence intervals). The finding of positive hares in each of the six geographic regions surveyed suggests that *L. infantum* is widely spread in Spanish hare populations. These findings together with the recent evidence of the ability of *Ph. perniciosus* to get infected through feeding on *Le. granatensis* [7], evidences the reservoir potential of hares for *L. infantum*. Infection by *L. infantum* in Iberian hares seems not to cause clinical disease [7] and thus *L. infantum* may not be of direct concern for hare conservation purposes. However, since *L. infantum* is an important pathogen for humans and other mammals, animal health and conservation authorities in Spain should be aware of the indirect consequences on conservation and wildlife management caused by their potential role as *L. infantum* reservoir.

Both Iberian and European hares are widely distributed in Spain, and their impact in the epidemiology of Mediterranean leishmaniasis deserves further research. Our findings suggest that hares have the potential to modulate the ecology of *L. infantum* in the near future as already evidenced in the outskirts of Madrid. The European hare inhabits vast areas of central Europe [13], constituting a potential European reservoir for *L. infantum*. This should be carefully considered when modeling the spread of *L. infantum* in those areas. Translocation of European and Iberian hares for hunting purposes between European countries is frequent e.g. between Spain and France [14,15]. This increases the chance of introducing *L. infantum* to new areas or other European countries via infected hares. It could also explain the great diversity of RFLP patterns found in European hares from the Atlantic region in Spain, where introduction of animals from French farms is common [15]. Moreover, translocation of hares at the national scale is frequent, which could explain the finding of similar PCR-RFLP patterns from hares surveyed in different geographic regions. Otherwise, our findings would indicate the existence of a widespread pattern of *L. infantum* strains in hares across Spain.

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