**Leishmania in wolves in northern Spain: A spreading zoonosis evidenced by wildlife sanitary surveillance**

# Leishmania in wolves in northern Spain: A spreading zoonosis evidenced by wildlife sanitary surveillance

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## A B S T R A C T

Leishmaniosis is, to date, considered the second most important emerging vector-borne protozoal disease in the world after malaria. The form of zoonotic visceral leishmaniosis found in the Mediterranean basin is caused by Leishmania infantum, and its life cycle includes the domestic dog and a phlebotomine sandfly vector. This complex epidemiological cycle and its high prevalence of subclinical infection, hinder the surveillance and control of L. infantum, and allows it to go unnoticed at the geographical endemicity limits of the parasite or in recently colonized areas. We, therefore, tested 102 wolves (Canis lupus) and 47 other wild carnivores in order to detect Leishmania DNA by means of PCR. Samples were collected from 2008 to 2014 in Asturias (northern Spain), a region considered non-endemic for the parasite. The results obtained provided valuable information regarding the prevalence of Leishmania in wild carnivores in Asturias and its geographic distribution in the region: an average prevalence of 33% for wolves and an overall prevalence of 40% for all the wild carnivores studied were reported, with a widespread presence of the parasite in the region and an apparent increase in its prevalence in wolves during the last decade. This suggests the usefulness of the wolf as a sentinel species for the detection and study of Leishmania in the field and confirms the value of wildlife sanitary surveillance programs for the de- tection and monitoring of hitherto disregarded diseases that affect domestic animals and humans.

## 1. Introduction

Leishmaniosis is, to date, considered the second most important reemerging vector-borne protozoal disease (after malaria), with 350 million people at risk of contracting it and some 2 million new human cases occurring every year, principally in developing countries (WHO, 2010). Zoonotic visceral leishmaniosis is the typical and most dan- gerous (lethal when untreated [Dujardin et al., 2008]) form of this parasitosis in the Mediterranean Basin. This zoonosis is caused by Leishmania infantum, which is the most frequent cause of cutaneous leishmaniosis in this region. Zoonotic visceral leishmaniosis has been endemic to southern Europe for decades, and is also potentially fatal to dogs, which are considered the main reservoir of infection. L. infantum requires the participation of a phlebotomine sandfly vector to complete its life cycle (namely Phlebotomus perniciosus and Phlebotomus ariasi in the Iberian Peninsula, Lucientes et al., 2005; Maroli et al., 2012). The

implication in this life cycle of a mammal host, an arthropod vector and all the factors affecting them from an epidemiological point of view makes the management and control of leishmaniosis a challenging task (Solano-Gallego et al., 2011). The sexual, vertical and iatrogenic transmissions of L. infantum have also been reported in dogs (Morillas- Marquez et al., 2002; Pangrazio et al., 2009; Silva et al., 2009), al- though usually as anecdotal events with an uncertain epidemiological relevance.

The development of molecular techniques, such as PCR, during the last few decades has made it possible to demonstrate that the pre- valence of the infection in dogs is much higher (often exceeding 60% of the population in endemic areas) than the proportion of animals that develop apparent signs of the clinical disease (Solano-Gallego et al., 2001, 2011).

Although the domestic dog is considered to be its main vertebrate host, Leishmania infection has also been confirmed in several wild and

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zoo mammal species throughout the world (Domingues Souza et al., 2014; Millán et al., 2014a). Given its taxonomic closeness to the dog, the wolf (Canis lupus Linnaeus 1758) is probably the most suitable "alternative host" for Leishmania in the wild, and it has already been confirmed to share several pathogen agents with sympatric dogs in the

study area (Oleaga et al., 2011, 2015; Millán et al., 2016).

The Autonomous Community of Asturias (northern Spain) is, to- gether with the bordering Communities of Galicia, Castilla y León and Cantabria, home to the largest population of wolves in Western Europe, and a population of more than 2000 individuals was estimated in this area in 2008 (Blanco et al., 2008). The detection of diseases or pa- thogen agents in the wolves studied in Sanitary Surveillance Programs provides valuable information for wolf management and conservation, and it has also been suggested that it may be an interesting source of information as regards the different pathogens shared with sympatric

Table 1

Number of individuals sampled (N) and Leishmania PCR prevalence values (including 95% confidence interval) in carnivores other than wolves studied in Asturias (Spain) from 2008 to 2014.

| Species | N | PCR positives % | 95% confidence interval |
|---|---|---|---|
| Red foX (Vulpes vulpes) | 13 | 46 | 23, 71 |
| Otter (Lutra lutra) | 10 | 70 | 40, 89 |
| Pine marten (Martes martes) | 8 | 62 | 31, 86 |
| Dog (Canis familiaris) | 4 | 25 | 5, 70 |
| Genet (Genetta genetta) | 4 | 75 | 30, 95 |
| Wild cat (Felis silvestris) | 3 | 100 | 44, 100 |
| Stone marten (Martes foina) | 3 | 67 | 21, 94 |
| Pole cat (Mustela putorius) | 3 | 0,00 | 0, 56 |
| Stoat (Mustela erminea) | 2 | 0,00 | 0, 66 |
| Badger (Meles meles) | 1 | 0,00 | 0, 79 |
| Total | 51 | 53 | 40, 66 |

domestic animals and even humans (Aguirre, 2009; Millán et al.,

2014b).

Asturias is the Spanish Community for which the lowest incidence of human leishmaniosis was reported in the period 2000–2010, and only one of the 6220 hospital admissions reported in Spain by the CMBD (Conjunto Mínimo Básico de Datos/Minimum Basic Data Collection – Amela et al., 2012) was located in that area in the decade in question.

This region, which is located in northern Spain, continues to be con- sidered a non-endemic area for canine leishmaniosis (Solano-Gallego et al., 2011), with zero or very low average seroprevalence values (< 5%) obtained for the dogs in the study area (Miró et al., 2012, 2013). Nevertheless, the detection of L. infantum in wild canids (Sobrino et al., 2008; Oleaga et al., 2015) and even in other species, such as hares (Ruiz-Fons et al., 2013), in Asturias during recent years highlighted the need for further studies focused on the distribution and epidemiology of the parasite.

The aim of the present study was to assess the presence and dis- tribution of L. infantum in wild carnivore species in Asturias in order to explore the knowledge regarding the epidemiology of the disease, and the role that wild species may play in it, in greater depth. The tax- onomical closeness of the wolf to the dog, together with the widespread distribution of this wild canid in Asturias and the wildlife sanitary surveillance program in which it is included, led us to propose its usefulness as a sentinel species for the detection of this protozoan in rural areas.

## 2. Materials and methods

### 2.1. Study area

The study was carried out in the Principality of Asturias, a region covering of 10,603 km2 located in northwestern Spain and bordered by the Bay of Biscay to the north and the Cantabrian Mountain chain to the south. The mountainous interior topography of the region, together with the influence of the gulf stream, causes the predominance of a temperate oceanic climate (Peel et al., 2007), with abundant rainfall evenly distributed throughout the year, and a mild average seasonal temperature, even in winter. These conditions favor the development of vegetation composed of deciduous and miXed forests interspersed with open pastures and meadows.

The wolf currently occupies almost the whole territory of the Principality of Asturias, with the exception of the central Region, which is mostly populated by humans (Blanco et al., 2008; García and Llaneza, 2012). The region can be divided into three different geographical areas: western, central and eastern Asturias, which are separated by large north-to-south oriented valleys running through the Cantabric mountain range. The estimated average densities of wolves in the western, central and eastern areas in 2010 were 2.2, 2.3 and 1.1/ 100 km2, respectively (when considering an average winter pack size of

4.93 wolves in the Cantabrian chain [García and Llaneza, 2012]). Limited data is available as regards the current distribution of the other

carnivores sampled, whose abundance, and in most cases also their distribution area, is anyway wider than that reported for wolves in Asturias (Palomo et al., 2008).

### 2.2. Sampling

The animals surveyed in the present study were 102 wolves and 47 small carnivores belonging to nine species (Table 1) submitted for ne- cropsy between January 2008 and May 2014 as part of the wildlife sanitary surveillance program established in Asturias. Four sympatric dogs submitted for necropsy from 2013 to 2014 were also included in the study.

The causes of death included population control hunts carried out by wildlife officers (85 wolves), vehicle collisions (13 wolves and 31 small carnivores), sarcoptic mange (6 red foXes [Vulpes vulpes]) and poison (2 dogs). It was not possible to determine the cause of death of 4 wolves, 10 small carnivores and 2 dogs.

The inclusion of the 47 small carnivores in the study allowed a more complete assessment to be made of the geographical distribution of the parasite in Asturias (see Fig. 1). It was not possible to register complete data for nine small carnivores in order to discover their circumstances and geographical origin owing to an incomplete submission of data.

The complete collection of data and tissue samples included the collection of spleen for the detection of L. infantum DNA using PCR assay. The samples were stored at −20 °C before being processed for PCR analysis. In addition to the samples collected, the size and char- acteristics of dermal and/or internal lesions were recorded, and samples were taken for histopathological examination when available from detected lesions. Although the exact time that elapsed between death and the necropsy of the animals found dead is unknown, no animals found in a bad state of preservation or a putrescent condition were included in this study.

## 2.3. PCR diagnosis

Total genomic DNA was extracted from each spleen sample (≈10 mg) using a commercial kit (GenEluteTM Mammalian Genomic

MiniPrep Kit, Sigma–Aldrich, MO, USA) according to the manu- facturer's instructions. A constant and highly specific fragment of L. infantum kDNA was amplified using an mRV1–mRV2 primer pair (Zanet

et al., 2014). Positive and negative control samples were included in each PCR assay and all standard precautions were taken to avoid con- tamination. PCR positive samples were purified and sequenced (Mac- rogen, Amsterdam, The Netherlands). The sequences obtained were compared to those available in GenBank in order to confirm the identity of each parasite.

Fig. 1. Distribution map of sampled (white figures) and PCR positive (black figures) wolves (circles), other small wild carnivore species (triangles) and domestic dogs (stars) studied in Asturias (northern Spain) for the detection of Leishmania DNA from 2008 to 2014.

## 2.4. Statistical analysis

Two different Generalized Linear Models (GLM; one analyzing the results obtained for wolves and the other analyzing those obtained for both wolves and the other sympatric wild carnivore species studied, Table 1) were developed in order to assess the factors potentially de- termining the presence of Leishmania. The PCR result was selected as a response categorical variable, whereas age, sex, area (western, central or eastern Asturias), cause of death and expected activity of the phle- botomine sandfly vectors at the moment of sampling (considering them to be active from May to October in the Iberian Peninsula, Lucientes- Curdi et al., 1991; Aránguez et al., 2014) were included as explanatory factors in both GLMs, using a binomial distributed error with a logistic link function. In the case of the wolf, the presence or absence of skin lesions reported during necropsy was also included as an explanatory factor.

The data was analyzed using the SPSS statistical package, version

17.0 (SPSS Inc., Chicago, IL, USA).

## 3. Results

The overall Leishmania prevalence value reported in the present study when considering all the animals studied was 40% (95% CI: 32–48%, n = 153), with a confirmed presence of the parasite DNA in 34 of the102 wolves studied (an average prevalence value of 33% in

wolves, 95% CI: 25–43%) and in 27 of the other 51 carnivores sampled in the study area (an average prevalence value of 53%, 95% CI: 40–66%), including one positive dog out of the four studied (Table 1). The sequencing of positive samples confirmed the identification of L.

infantum. All analyzed samples had high sequence homogeneity (> 98%) with the consensus L.infantum sequence.

The geographical distribution of the sampled and positive animals is shown in Fig. 1. The annual variation in the number of wolves that were PCR positive to L. infantum is reported in Fig. 2, whereas Table 2 de- scribes the distribution by the site, sex and age of those wolves with a confirmed presence of Leishmania DNA.

Regarding the presence of Leishmania DNA in carnivore species other than the wolf, the overall average prevalence reported was 53% (n = 51). In the case of those small carnivores whose submission data were complete, including the sampling location (n = 42), the pre- valence was 33.3%, 50% and 54.5% for western, central and eastern Asturias, respectively. The average prevalence values obtained for these

Fig. 2. Inter-annual variation of average PCR-based prevalence (+ standard error represented in error bars) of Leishmania throughout the study period (2008–2014) in wolves studied in Asturias.

* = Prevalence data regarding Leishmania in wolves in Asturias prior to 2008

(←2008) taken from the study of Sobrino et al. (2008), who studied the prevalence of Leishmania in Spanish wild carnivores from 1990 to 2008 (PCR prevalence reported in Asturian wolves = 18.1%, n = 33).

species are reported in Table 1. Leishmania DNA was, for the first time to the best of our knowledge, detected in wild otters (Lutra lutra).

The only macroscopical lesions detected in the present study that could be related to leishmaniosiswere the dermal lesions reported in several wolves since 2008. Performed histological studies confirmed those skin alterations to be mange lesions (Oleaga et al., 2011), while no other leishmaniosis-compatible lesions were detected in the internal organs studied.

No statistically significant relationship was detected between the presence of Leishmania DNA and the age, sex, expected activity of sandflies at the moment of sampling, geographic origin or the cause of death of the animals studied. In the case of the wolf, no relationship was detected between the presence of the skin lesions reported at necropsy and the detection of Leishmania by PCR.

## 4. Discussion

The present study is, to the best of our knowledge, the most com- prehensive one carried out on the prevalence of Leishmania in wild

Table 2

Prevalences based on PCR detection of Leishmania infantum in wolves in Asturias (2008–2014), including distribution by geographic area, sex and age. Sample sizes are indicated in parenthesis (n), and 95% confidence intervals in italics beneath.

| Age | Western area | Central area | Eastern area | ♂ | ♀ | Total | |
|---|---|---|---|---|---|---|---|
| Pups | 25% (12) 9, 53 | 33.3% (9) 12, 65 | 33.3% (6) 10, 70 | | | 33.3% (15) 15, 58 | 25% (12) 9, 53 |
| | 29.6% (27) 16, 48 | | | | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Yearlings | 35.3% (17) 17, 59 | 22.2% (9) 6, 55 | 30% (10) 11, 60 | 22.7% (22) 10, 43 | 42.8% (14) 21, 67 | 30.5% (36) 18, 47 | | | | | | |
| Adults | 40% (15) 20, 64 | 47% (17) 26, 69 | 14.3% (7) 3, 51 | 34.8% (23) 19, 55 | 43.7% (16) 23, 67 | 38.5% (39) 25, 54 | | | | | | |
| Total | 34.1% (44) 22, 49 | 37.1% (35) 23, 54 | 26.1% (23) 13, 46 | 30% (60) 20, 43 | 38.1% (42) 25, 53 | 33.3% (102) 25, 43 | | | | | | |

wolves (with 102 individuals studied), and also showing the highest prevalence for this species to date (Mohebali et al., 2005; Sobrino et al., 2008; Sastre et al., 2008; Del Río et al., 2014). The results obtained show a high prevalence of Leishmania in apparently healthy wild car- nivores in Asturias, northern Spain. These prevalence data, together with the widespread geographical distribution of the parasite (Fig. 1) and the large number of species harboring the protozoan (Table 1), provide evidence of a widespread presence of the parasite. This is striking for a region previously regarded as non-endemic (Solano- Gallego et al., 2011).

The absence of significant differences or relationships between the presence of Leishmania DNA and the age, sex or geographic origin of the animals studied has been reported in other wild carnivore species such as the red foX in both northern Spain (Sobrino et al., 2008; Del Río et al., 2014) and a leishmaniosis-endemic area in southern Italy (Dipineto et al., 2007). Nevertheless, Verin et al. (2010) reported a statistically significant higher rate of infection for mature foXes when compared to young ones (< 1 year) in another endemic region in central Italy.

The prevalence values in Asturian wolves are lower than those re- ported for other wild canids, such as the red foX, sampled in the areas considered endemic for L. infantum in central Spain (Criado-Fornelio et al., 2000) and Southern (Dipineto et al., 2007) and central (Verin et al., 2010) Italy, whereas very similar (Del Río et al., 2014) or lower (Sobrino et al., 2008) values were reported for red foXes in northern Spain in previous studies. It is interesting to emphasize that the average prevalence of 33% in wolves from Asturias is higher than the 9% re- ported for red foXes sampled in Southern France, where zoonotic leishmaniosis is endemic (Davoust et al., 2014). In this respect, the greater size of the territories occupied by wolves and their capacity to cover long distances should be taken into account.

With regard to the comparison of the data obtained with those

available for dogs, they remain within the range found in domestic dogs in endemic areas in Spain using PCR (Solano-Gallego et al., 2001; Fisa et al., 2001). Dogs had average seroprevalences of 4.7% and 0%, along with low antibody titers against Leishmania in serologic surveys carried out in Asturias (Miró et al., 2012, 2013) respectively. No specific study evaluating the distribution and prevalence of Leishmania in Asturian dogs (using PCR or qPCR) has, to the best of our knowledge, been published to date. The seroprevalence data reported for dogs in the area agree with the extremely small number of dogs with confirmed leish- maniosis in Asturias to date (often as anecdotal non-autochthonous cases) and support the fact that Asturias is considered a non-endemic region (Solano-Gallego et al., 2011) for this parasite. Nevertheless, the infection rates in wild carnivores obtained in the present study high- light an important presence and widespread distribution of this parasite in Asturias and an apparent increase in its prevalence in wolves when compared with previous studies carried out in the same region (Sobrino et al., 2008). A northward spread of this protozoan has been described during the last few decades in other regions at the northernmost limit of leishmaniosis endemicity in Europe, including Italy (Maroli et al., 2008), France

(Chamaille et al., 2010) and northern Spain (Amusategui et al., 2004), where climatic factors interacting with human behavior have been proposed as important causes (Ferroglio et al., 2005; Menn et al., 2010).

The prevalence data obtained for the red foX, otter, pine marten, stone marten, genet and wildcat were similar to or even higher than those reported for the wolf, and also higher than those reported in previous studies dealing with wildlife in northern Spain (Sobrino et al., 2008; Del Río et al., 2014). Considering the average size of the terri- tories occupied by these small wild carnivores, which are usually smaller than those occupied by wild wolves, the results obtained highlight an apparent widespread distribution of L. infantum. Apart from the detection of Leishmania infection in wild otters (for first time, to the best of our knowledge), the high prevalence detected in wildcats is probably the most outstanding result as regards a wild carnivore species other than the wolf in the present work. Although based on such a small sample size, the fact that the three wildcats examinied tested positive to Leishmania reveals the need to include both wild and do- mestic cats in surveillance programs in order to better understand their role in the parasite´s epidemiology (Pennisi et al., 2015).

Despite the high average prevalence reported as regards Leishmania

in wild carnivore species, no clinical case (disease) attributed to this parasite has been confirmed in wildlife in Asturias to date. This natural infection by Leishmania in apparently healthy populations has already been described in several wildlife species in Europe (Millán et al., 2014a), including several wild carnivore species in areas endemic to Spain, Italy and France (Criado-Fornelio et al., 2000; Verin et al., 2010; Davoust et al., 2014), along with periendemic areas of northern Spain (Del Río et al., 2014). The fact that no statistical relationship was de- tected between the presence of skin lesions and the detection of Leish- mania DNA from the wolves studied coincides with previous etiological and histopathological findings showing that Sarcoptes scabiei caused the skin lesions reported in wolves in Asturias since 2008 (Oleaga et al., 2011, 2012). Keeping in mind the proposition that lesions on skin harboring amastigotes facilitate the transmission of Leishmania by sandflies (Millán et al., 2014a), sarcoptic mange should be taken into account not only as a possible immunosuppressive concomitant process, but also as a fur-injuring illness facilitating the access of sandflies to this protozoan. The high prevalence of Leishmania in wild carnivores in Asturias suggests the advisability of including canine leishmaniosis in the list of differential diagnoses to be considered in wolves with skin lesions or any other alteration compatible with the disease. It also highlights the need to redefine the way in which camera-trapping can be used as a field technique for the surveillance of sarcoptic mange (Oleaga et al., 2011) and other diseases producing skin lesions.

A previous study carried out in Asturias reported the absence of

antibodies against Leishmania in 39 wolves analyzed, despite 18 of them were positive for the presence of Leishmania DNA (Oleaga et al., 2015). This result coincides with previous data reported for 33 captive wolves in southern Europe (Sastre et al., 2008) and agrees with the asympto- matic condition of the wolves studied. The absence of a confirmed clinical disease related to Leishmania in the more than 100 wolves analyzed in the present study suggests that this parasite does not mean, at least at the present date, a threat to the maintenance of Asturian wolf populations.

The virtually zero detection of P. ariasi and P. perniciosus in Asturias at present, with only one single P. ariasi male captured in 2006 (Javier Lucientes, personal communication) coincides with the fact that Asturias is considered a non-endemic area for leishmaniosis (Solano-

Gallego et al., 2011), but conflicts with prevalence data reported for Asturian wild carnivores in the present study. Some aspects, such as i) the immigration of infected wolves from adjacent endemic areas, ii) the

participation of unknown or “permissive” vectors in a possible sylvatic

cycle, or iii) a higher than usually reported (Díaz-Espińeira and Slappendel, 1997; Duprey et al., 2006) relevance of infection without the involvement of sandflies (i.e., via bite wounds, Karkamo et al., 2014), cannot be completely ruled out before further research, but seem unlikely explanations for the results obtained. It has been proposed that climatic change affects the density, dispersion and activity patterns of sandflies, thus influencing the distribution and epidemiology of dis- eases owing to the pathogens that they transmit (Maroli et al., 2008, 2012). An increase in the density and distribution area of competent vectors for Leishmania (namely P. ariasi) in Asturias in recent years may not yet have been detected owing to the limitation of specific surveys. One possible explanation for the high prevalence reported for wild carnivores in Asturias is the existence of a sylvatic cycle. The molecular differences between Leishmania strains from dogs and wildlife reported in previous studies support the predominant role of the latter in a syl- vatic cycle of leishmaniosis (Sobrino et al., 2008; Millán et al., 2011), suggesting independent domestic and sylvatic cycles (Del Río et al., 2014). Its relatively high densities and taxonomic closeness to the dog and wolf signify that the red foX has usually been proposed as a key piece in the maintenance of a sylvatic cycle of Leishmania (Millán et al., 2014a). However, the small number of samples available in this study prevented a real assessment of the current prevalence in red foXes in the

study area

The detection in the present study of an autochthonous case of Leishmania infection in a sheepdog (Table 1) highlights the possible participation of rural dogs in this sylvatic cycle or in the interaction between a sylvatic and a domestic cycle maintained by dogs (Quinell and Courtenay, 2009). Dogs living in rural areas (and in Asturias, these are mainly hunting and sheepdogs) tend to live outdoors, and share their habitat and exposure conditions with the pathogens and sandflies (Miró et al., 2013) of sympatric wild carnivores (Millán et al., 2016). The high prevalence of subclinical infection of canine leishmaniosis (Baneth et al., 2008; Solano-Gallego et al., 2011), the wide variety of

clinical signs that this disease can show (Solano-Gallego et al., 2009) and the “non-endemic” consideration of Asturias for the disease (WHO, 2010; Solano-Gallego et al., 2011) could explain its absence in the list of differential diagnostic tests for local practitioners and a possible un- derestimation of the real distribution of the parasite and the situation of

the disease in dogs in Asturias.

## 5. Conclusion

The use of molecular techniques for the detection of Leishmania DNA in 147 wild carnivores, including 102 wolves, sampled since 2008 provided valuable information about the prevalence and geographic distribution of Leishmania in wild carnivores in Asturias, showing a high prevalence and a widespread presence in the region. The data obtained suggest an apparent increase in the prevalence in wolves from the area during last decade and the possible exposure of rural dogs to the parasite. The present study also suggests the usefulness of the wolf as a sentinel species for the detection and study of L. infantum in the field and confirms the value of wildlife sanitary surveillance programs for the detection and monitoring of certain neglected pathogen agents that can affect wildlife, domestic animals and humans.

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