

Detection of *Leishmania infantum* kinetoplast DNA by Real Time PCR in hair of wild rabbits

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Summary

The study of potential wild mammal reservoirs is necessary for the surveillance of leishmaniasis, as *Leishmania* protozoans have been isolated from a wide range of wild and domestic animal species, including *Leporidae*. Recently, it has been demonstrated that both hares and wild rabbits can act as sylvatic reservoirs of *Leishmania*. In Spain, most of the research involving wild rabbits has been developed in the central area of Madrid and in the southeastern Mediterranean coast. We studied the presence of *Leishmania infantum* in 116 wild rabbits (*Oryctolagus cuniculus*) captured in Santovenia de Pisuerga, Valladolid, Spain. Hair samples were analyzed by real time PCR. *L. infantum* kinetoplast DNA (kDNA) was detected and quantified in 4 out of 116 analyzed animals. The estimated number of parasites obtained were quite variable, ranging from 2.60 to 276.60. Hair samples can be collected by non-invasive methods, being a proper sample for *Leishmania* detection in wild *Leporidae*, which have an important role as reservoirs of *Leishmania*. Our findings enhance the need for more extensive studies in different geographical areas.

Keywords: hair, *Leishmania infantum*, rabbit, kDNA, Real Time PCR

Leishmaniasis is a worldwide zoonoses caused by protozoan *Leishmania*, being *Leishmania infantum* the major cause of leishmaniasis in dogs and immunocompromised people in the Mediterranean area (27).

Leishmania parasites have been identified in a large number of wild animal reservoirs, where *Phychodidae* insects of the genus *Phlebotomus* and *Lutzomyia* act as vectors (3, 27). A wide range of wild mammals from different orders are adapted to infections by *Leishmania* spp. (4), contrary to dogs and humans (1, 26). Most of the wild reservoirs for all *Leishmania* species belong to the *Rodentia* order (8).

Lagomorpha order became a target due to an epidemic outbreak in Madrid (Spain) affecting about 500 immunocompetent people with both skin and visceral clinical expressions from 2009 to 2020 (2, 18). *Leporidae* (especially *Oryctolagus cuniculus* and *Lepus granatensis*) were the reservoirs responsible for the transmission of *L. infantum* to *P. perniciosus* pres-

ent in the area (18, 20). *Leporidae* are highly adapted to *Leishmania* infection and do not develop a visceral form of the disease as seen in canids or humans (8). Recent studies in rabbits revealed no histological lesions typical of leishmaniasis, as well as low parasitic loads. The skin was proven as the organ with the highest parasite load, indicating its importance for both diagnosis and transmission to sand flies (18, 24). Xenodiagnostic studies demonstrated the ability of hares to transmit *L. infantum* to *P. perniciosus* (18, 20) and the detection of *L. infantum* in *P. perniciosus* from the same leishmaniasis focus in Spain (17, 14, 15).

Serological diagnostics methods as ELISA, DFA and IFAT have been performed for the diagnosis of leishmaniasis from rabbits and hares, as well as parasitological methods such as culture and real time PCR in spleen, liver and skin (9, 11, 21). Recently it has been demonstrated that hair samples can be used for the molecular diagnosis of both *L. infantum* and *L. ma-*

for, and be employed in epidemiological, clinical, and experimental infections (5, 16, 22). In addition, hair is easy to obtain, causing no harm to the animals, and *Leishmania* kDNA can remain stable inside hair over time and after extreme environmental conditions (22).

The study of wild mammal reservoirs is essential for leishmaniosis surveillance. In Spain, most of research involving wild rabbits was developed in the central area of Madrid and in the southeastern Mediterranean coast. In the area of Castilla and León (North Plateau, Spain), the number of human cases were 15 from 2014 to 2018 (0.13-0.2/100.000 inhabitants) (19). Environmental changes such as disappearance of agriculture, livestock and traditional land uses, the presence of grass and shrubs, absence of predators and competitors and warmer temperatures facilitate the life and reproduction of rabbits and hares (2).

Taking these into account, the aim of this study was to determine by qPCR the presence of *Leishmania* kDNA in wild rabbits that could be reservoirs of the disease in an area of the province of Valladolid in Castilla and León (North Plateau, 190 km northwest of Madrid), close to Santovenia de Pisuerga, a recently changed village of around 5.000 inhabitants where shrubs and grass have covered extensive areas mixed with sandy zones very auspicious for sandflies spreading.

Material and methods

Animals. A sample of 116 wild rabbits was captured between May 2017 and March 2018 in Santovenia de Pisuerga, Valladolid, Spain (ETRS89/UTM Zone 30). The sampling area was divided in 5 zones: Zone 1 (Z1: 19 animals), Z2 (28 animals), Z3 (28 animals), north (Zn: 22 animals) and south (Zs: 19 animals) (Fig. 1). The sampling zones were selected due to changes in their agricultural and livestock use as well as their environmental conditions: shrubs and grass mixed with sand areas. Animals from Z1, Z2, and Z3

could be in contact with each other; animals from Zn and Zs cannot be in contact neither between them nor with the other zones due to the large distances. The animals were captured by ferret hunting (10) under the authorization of the Public Health authorities of the Community of Castilla y León, sedated and euthanized following the national RD 53/2013 and the European Directive 2010/63/EU. Rabbits were transported to the lab within the first 5 h after their capture and were necropsied in a biological safety cabinet. Macroscopic lesions compatible with subclinical infections were not observed.

Samples. A number of 50-100 hairs were taken with scissors (avoiding wounds and/or blood spots) from two corporal zones (back and flank), introduced into sterilized plastic bags, and sent to the laboratory. Samples from all rabbits were stored at -80°C until DNA extraction.

DNA extraction and qPCR analyses. About 20 hairs from each animal were introduced in screw cap sterile microtubes and incubated overnight at 56°C in 250 μl of lysis buffer (10 mM TrisCl, 0.1 M EDTA, 0.5% SDS) with 100 mg/ml of Proteinase K. DNA was extracted using 200 μl of supernatant obtained from processed samples with the GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The qPCR technique used in the present study was performed following previously published protocols (6). Briefly, PCR reactions were carried out in 96 wells plates in a final volume of 20 μl (4 μl of DNA + 16 μl of Reaction Mix), containing 20 μM of each primer (Leish 1: 5'-AACTTTTCTGGTCCCTCCGGGTAG-3' and Leish 2: 5'-ACCCCCAGTTTCCCGCC-3'), 10 μM of TaqMan Probe (FAM-5'-AAAAATGGGTGCAGAAAT-3'-MGB), and the iTaq Universal Probes Supermix (Biorad Laboratories, Hercules, CA, USA). The thermal cycling profile used was one incubation step at 50°C for 2 min and an initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing-extension at 60°C for 1 min. Each amplification run contained positive and negative controls. DNA extracted from 55.000

L. infantum promastigotes MCAN/ES/1996/BCN150 zymodeme MON-1 was used as a positive control. DNA extracted from healthy dog blood samples was used as negative control. All analyses were performed in a Step One Plus Real Time PCR System (Applied Biosystems Laboratories, Foster City, CA, USA). To quantify *Leishmania* kDNA, a standard curve was carried out using DNA extracted from six quantities of *L. infantum* parasites (MCAN/ES/1996/BCN150, zymodeme MON-1) ranging from 50.000 to 0.5 (dilution factor $\times 10$); DNA was extracted and analyzed together with the hair samples in triplicate. The threshold cycle (Ct) corresponding to the Y-intercept of each analysis (that is, the expected Ct value for the estimated quantity of 1 parasite) was used as cut-off, positive being those

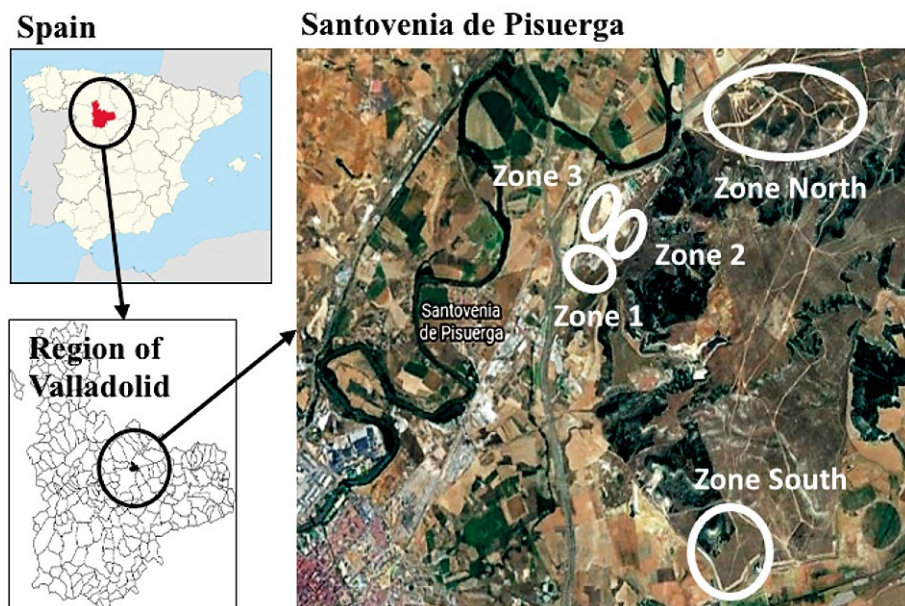


Fig. 1. Geolocation of the areas where wild rabbits were captured for *Leishmania* detection. Areas in Santovenia de Pisuerga, Valladolid, Castilla and León, Spain

samples whose Ct values were \leq Y-intercept value of each assay. Samples were considered positive when the Ct of each one of the triplicates was lower than 32.

Results and discussion

Table 1 shows qPCR results of hair analysis (from back and flank) of wild rabbits from Valladolid province. The qPCR method was able to detect and quantify the presence of *L. infantum* kDNA in 4 out of 116 (3.5%) analyzed animals. The estimated number of parasites in positive samples were quite variable, ranging from 2.60 to 276.60. The highest quantity of parasite DNA was observed in the Z1 geographical area, being the estimated number of parasites in the hair of one individual (No. 15) up to 100 times greater than that observed in other animals. The results from animal No. 12 gave a Ct value (32.15) close to the threshold (32.00) but it was considered as negative. No positive results were found in animals from Z2, Z3, Zn or Zs areas. The reliability of the results obtained was assessed by the R^2 coefficient data (from 0.996 to 0.999), slope (from -3.36 to -3.33) and the high efficiency reached (between 98.56% and 99.64%). Hair samples can be obtained by non-invasive methods in wild animals and they are stable to the environmental conditions of storage and transport (7, 12, 13). Moreover, hair protects parasitic DNA from external degradation, storage at -80°C , desiccation and UV radiation (22). Our results demonstrate that hair is a proper sample for *Leishmania* detection in wild *Leporidae*.

The variability observed among animals regarding parasite load was in accordance with other studies previously published where scarce positive animals and low clinical impact were described (11, 23, 24). Ortega et al. (23) studied the presence of *L. infantum* in leporids detecting for the first time *L. infantum* DNA in 67% (22/33) and 53% (17/32) of hair samples from rabbits and hares, respectively. These authors also found that the 100% (7/7) of rabbits and 33% (1/3) of hares were positive to the presence of *L. infantum* in all samples studied: spleen, skin and hair. This could be due to the fact that wild mammals would act as asymptomatic reservoirs since no macroscopic lesions were detected in post-mortem examination of positive animals (24). In the present study, no macroscopic lesions were found during the necropsy of the rabbits. Some authors (21, 25) reported that the absence of lesions could be due to (i) a recent association between the host and the parasite, (ii) to a low number of parasites or (iii) a reduced virulence of the parasite maximizing its transmission.

The knowledge of the ecology of leishmaniosis requires hard work to identify the hosts involved in

Tab. 1. Real Time PCR results of the analysis of hair (from back and flank). A positive PCR result was considered when the Ct mean (from triplicate analyses) was lower than 32. No positive results were found in animals from Z2, Z3, Zn or Zs areas. ND, not detected

| Area | No. of animals | No. of positives | Animal No. | Ct mean | Mean of estimated no. of parasites |
|------|----------------|------------------|----------------|---------|------------------------------------|
| Z1 | 19 | 4 | 1-4, 8, 10, 14 | ND | – |
| | | | 5 | 36.75 | – |
| | | | 6 | 31.14 | 2.60 |
| | | | 7 | 37.68 | – |
| | | | 9 | 39.78 | – |
| | | | 11 | 38.40 | – |
| | | | 12 | 32.15 | – |
| | | | 13 | 35.93 | – |
| | | | 15 | 24.52 | 276.60 |
| | | | 16 | 27.38 | 36.45 |
| | | | 17 | 34.03 | – |
| | | | 18 | 29.46 | 8.67 |
| | | | Z2 | 28 | 0 |
| 1 | 35.61 | – | | | |
| 2 | 38.95 | – | | | |
| 14 | 38.76 | – | | | |
| 16 | 38.72 | – | | | |
| 17 | 36.91 | – | | | |
| 19 | 36.88 | – | | | |
| Z3 | 28 | 0 | 2-28 | ND | – |
| | | | 1 | 38.34 | – |
| Zn | 22 | 0 | 1-22 | ND | – |
| Zs | 19 | 0 | 1-19 | ND | – |

the disease in different geographical areas. The studies involving wild mammals need costly techniques based on *post mortem* analysis or *in vivo* sampling (22). Xenodiagnostic studies already performed with wild rabbits and Iberian hares proved that these mammals are able to transmit *L. infantum* to *P. perniciosus* being strong evidence of its role in *Leishmania* transmission even those without clinical signs (20).

In Spain, *L. infantum* has been investigated in wild rabbits and hares in two geographical areas, i.e., Madrid (2, 11, 18, 23) and the southeastern Mediterranean coast (9). In the present study, the sampling area was close to a village with recently changed country areas that could promote the spreading of sand flies and rabbits.

Our results demonstrate that hair is a proper sample for *Leishmania* detection in wild *Leporidae*, as it has also been proven in other wild mammals. The results of the present study show the important role of wild *Leporidae* – rabbits in the present work – as reservoirs of *Leishmania*, leading to the need for more extensive studies together with an entomological survey to elucidate the possible role of wild rabbits in a sylvatic cycle of transmission of *L. infantum* in this area.

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