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Antibodies to *Leishmania* in naturally exposed domestic ferrets (*Mustela putorius furo*) in Spain

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Highlights

- In endemic areas, it is possible to detect *L. infantum* seropositive healthy ferrets.
- Seasonal variation in anti-*Leishmania* antibodies can be detected in ferrets.
- Interpretation of specific antibodies is influenced by sand fly transmission period.

ABSTRACT

Zoonotic leishmaniosis due to *Leishmania infantum* is a vector-borne disease endemic in southern Europe and dogs are the main reservoir for this infection. Seasonal variations in antibody titers in this species in areas where phlebotomine vectors have seasonal patterns of activity are important for epidemiological, preventive and clinical studies related with canine leishmaniosis. It has been suggested that cats, rabbits and ferrets may act as peridomestic reservoirs and not only as accidental hosts. The aim of this study was to determine if seropositive ferrets (*Mustela putorius furo*) to *Leishmania* could be affected by seasonal variations of anti-*Leishmania* antibodies. A group of seropositive clinically healthy ferrets (n=21) were included in this study. A significant reduction in anti-*Leishmania infantum* antibodies was detected during non-transmission period (December 2020-February 2021) in comparison to transmission period (April-October 2020). This study describes for the first time a seasonal variation in the anti-*Leishmania* antibodies detected in domestic ferrets following natural exposure during sand fly transmission period and the following non-sand fly transmission period in a Mediterranean area considered as an area where *L. infantum* is endemic.

Keywords: antibody; ferret; *Leishmania infantum*; *Mustela putorius furo*.

1. Introduction

Leishmaniosis is a vector-borne disease caused by *Leishmania infantum*, with the parasite being transmitted by phlebotomine sand flies under natural conditions. It is considered one of the most important vector-borne zoonoses in Europe (Baneth et al., 2016). Although various phlebotomine species are implicated in the transmission of *L. infantum* in Europe, only two of them are found in Spain: *Phlebotomus ariasi* and *Phlebotomus perniciosus* (Lucientes et al., 2005), with *P. perniciosus* being the prevalent vector in eastern Spain with a seasonal activity pattern from the end of March to November, with two peaks from June to July and from September to October (Lucientes et al., 2005).

Among pets, dogs are the primary domestic reservoir for human infection. However, other animals could be infected by the parasite, including domestic animals such as cats (Fernandez-Gallego et al., 2020). Recently, leishmaniosis has been diagnosed in a domestic ferret (*Mustela putorius furo*) using a wide range of confirmatory techniques (Giner et al., 2020). However, there is no information about the detection of anti-*Leishmania* antibodies in ferrets. In dogs, seroconversion after *Leishmania*-infected female *Phlebotomus* spp. bites is variable from 1 to 22 months, being shorter in experimental infection in comparison to natural infection (Moreno and Alvar et al., 2002). By contrast, timing of seroconversion is unknown for cats when the causative agent is *L. infantum*, whilst experimental infection with *Leishmania braziliensis* has shown that skin lesions usually occur before antibody response, and seroconversion can be detected when the lesions are improving and the size of the lesions decreased (Simoes-Mattos et al., 2005).

In areas where *L. infantum* is endemic, the majority of dogs are exposed to infection and this exposure can be detected by serological methods based on detecting a specific antibody response against *L. infantum* (Baneth and Aroch, 2008). For dogs, enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescent antibody test (IFAT), and the immunochromatographic rapid tests represent the most common methods used for the detection of the anti-*Leishmania* antibodies (Maia and Campino, 2018). In the same way, the presence of anti-*Leishmania* antibodies in cats can be detected by IFAT, ELISA, direct agglutination test (DAT) and Western Blot (WB) techniques (Pennisi and Persichetti, 2018).

In this context, the detection of high antibody levels is often associated with a high parasitic load and disease in dogs (Reis et al., 2006). Conversely, low antibody levels in clinically normal dogs with a negative result on molecular and/or parasitological tests may indicate exposure without direct detection of the parasite or early stages of *Leishmania* infection (Paltrinieri et al., 2010). Changes in anti-*Leishmania* antibodies titers with a seasonal variation during transmission or non-transmission period have been observed in dogs in Spain (Acedo-Sanchez et al., 1998) and Italy (Cavalera et al., 2021), associated with the seasonal pattern of sand fly activity.

This study describes for the first time a seasonal variation in anti-*Leishmania infantum* antibodies detected by a quantitative serological test in seropositive healthy ferrets living in Valencia, an area where leishmaniosis is endemic.

2. Material and methods

2.1 Study area

All ferrets included in this study were from the Province of Valencia (39° 28′12.864”N, 0° 22′36.48”W), on the east coast of Iberian Peninsula, which is an area with a high prevalence of canine leishmaniosis.

2.1. Characterization of the ferrets under study

From a total of 330 ferrets analysed to detect the presence of anti-Leishmania antibodies during one year, only some animals fulfilled the following criteria: seropositive to *Leishmania* in the transmission period and a second serum sample of the same ferret obtained in the non-transmission period. Twenty-one ferrets seropositive to *Leishmania* were included in this study. A complete physical examination was carried out before sampling. For each ferret, serum samples were collected aseptically by cranial cava venipuncture in two different temporal points, one sample was obtained during sand fly transmission period (April- October 2020) and the second sample during sand fly non-transmission period (December 2020- February 2021). Data on age, gender, cohabitation with a dog and lifestyle were recorded. Separated serum were stored at -20ºC until processing. The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of University of Zaragoza (protocol code PI25/20).

2.2. Serology

Anti-*Leishmania* antibodies were detected by ELISA using a crude *L. infantum* antigen (MHOM/FR/78/LEM75 zymodeme MON-1), as described by Giner et al. (2020). Sera were diluted at 1/50 and a protein A peroxidase conjugate (dilution, 1/8,000; Pierce) was used. The cutoff was set to 0.200 optical density units (OD) (mean + 3 standard deviations of values from 20 indoor ferrets from

northern Spain). Each test included serum from a *L. infantum* confirmed sick ferret from Spain with a *L. infantum* isolation as positive control and serum from a healthy, non-infected ferret as negative control. Sera with an OD ≥ 0.700 were classified as high positive, with an OD ≥ 0.400 and < 0.700 as medium positive, and with an OD >0.200 and ≤ 0.400 as low positive. OD between results obtained during sand fly transmission period and sand fly non-transmission period were considered different when OD variation was greater than 10%. All samples and controls were analyzed in duplicate in the same ELISA plate to avoid inter- and intratest variability.

2.3. Statistical analysis

SPSS software 22.0 for Windows (IBM Corp., Armonk, NY, USA) was used for the statistical analysis. A Kolmogorov-Smirnov test was performed and this test determine that the data were not normally distributed. Comparison of anti-*Leishmania* antibodies levels between periods (transmission and non-transmission) was tested with Wilcoxon signed rank test. Association between variation in anti-*Leishmania* antibodies (stable/increased/decreased) and the recorded variables (age, gender, cohabitation with a dog and lifestyle) were analyzed. The significance of these differences was assessed using chi-square test. A P-value <0.05 was considered significant.

3. Results

3.1 Characterization of the ferrets under study

All the tested ferrets (5 females and 16 males) had a mixture of coat colors and no ferrets had been neutered surgically. The age of the ferrets ranged from 3 to 9 years old. These ferrets had neither clinical signs nor laboratory abnormalities compatible with clinical leishmaniosis. None of the ferrets had been treated with long-acting topical anti-parasitic repellent against sand flies during the entire season of risk of exposure. Among the 21 seropositive ferrets, 10 animals lived with a dog at the same place. Considering the lifestyle, nine animals had an indoor lifestyle, two animals had an outdoor lifestyle and the remaining ferrets had a mix lifestyle.

3.2 Variation of anti-Leishmania antibodies between the transmission period and the non-transmission period

Overall, 12 ferrets of 21 examined showed a decrease of ELISA OD during the non-transmission period compared with the values observed during the transmission one. Table 1 shows the *L. infantum* serological results obtained by ELISA. The OD during transmission period was 0.331 ± 0.118 (mean±standard deviation), being the maximum value 0.731 and the minimum value was 0.202. By contrast, OD during non-transmission period was 0.267 ± 0.098 with a maximum value of 0.471 and the minimum value of 0.145. No significant association was found between positivity for *L. infantum* infection and the factors evaluated ($P>0.05$). In contrast, a statistically significant association was found between OD positivity during transmission period and OD positivity during non-transmission period ($P=0.04$)

Considering antibody variation between sand fly transmission period and sand fly non-transmission period, five ferrets (23.8%) had a similar anti-

Leishmania antibodies during both periods. In general, during the transmission period 17 ferrets presented low positive values, three medium positive values and one ferret a high positive value. When comparing with the non-transmission period, a decrease in antibody titers was observed: seven ferrets became seronegative, 11 ferrets had a low positive and finally three ferrets had a medium positive titer.

During the non-transmission period, anti-*Leishmania* antibodies increased in four ferrets (19%). Two of them increased from low levels to medium levels, whilst in the remaining ferrets OD was slightly increased, but both animals were classified as low positive. Anti-*Leishmania* antibody levels decreased in 12 of 21 (57,14%), with seven becoming seronegative (initially low positive during transmission period), and in five ferrets with a low positive result in the transmission period, OD decreased in the non-transmission period being classified as low positive. During the transmission period, one ferret with a high positive result and another ferret with a medium positive result shifted into low positive results during the non-transmission period.

3. Discussion

To the authors´ knowledge, this study describes for the first time a seasonal variation in the anti-*Leishmania* antibodies detected in domestic ferrets (*Mustela putorius furo*) following natural exposure, including sand fly transmission period and the following non-sand fly transmission period.

Female *Phlebotomus* spp. feed on a variety of vertebrate reservoirs (Killick-Kendrick, 1999), including humans, livestock, dogs, wild rabbits, hares, rodents and cats, with variable impacts on the epidemiology of leishmaniosis. The

opportunistic feeding behavior of *P. perniciosus*, taking blood meals from a range of reservoirs, has been demonstrated in Menorca (De Colmenares et al., 1995) and other Mediterranean foci (Branco et al., 2013; Risueño et al., 2017).

There are some reports describing seasonal variations in anti-*Leishmania* antibodies from dogs. Reduction of anti-*Leishmania* antibodies has been detected within the same transmission season (Acedo-Sánchez, et al., 1998) or between transmission seasons (Cavalera et al., 2021). Our results reinforce the importance of considering an antibody reduction level in other animals such as ferrets between transmission and non-transmission periods with a significant difference (P=0.04).

A potential limitation of this study was the reduced number of animals included to extrapolate the results obtained to the general ferret population located in endemic areas of *L. infantum*. Studies with higher number of subjects are necessary to better establish the most adequate tools to be used in the context of this study. The seropositive ferrets were classified as clinically healthy animals according to the absence of laboratory abnormalities detected by routine red blood cell count and clinical chemistry. In this sense, for a better characterization of *Leishmania* infection, an additional confirmatory including a qPCR technique should be performed in bone narrow or lymph node samples. Nevertheless, the procedure to obtain these two different samples is difficult to be accepted by the owner when the animal is apparently healthy without clinical signs and laboratory alterations. The problem of the phenomenon of cross-reaction could be a common situation with other trypanosomatids, but according to our knowledge, all ferrets lived in Valencia, a region in Spain where *Trypanosoma cruzi* is not present and *L. infantum* is the parasite responsible for canine leishmaniosis in Europe.

During blood feeding, saliva inoculated stimulates a species-specific antibody response because this saliva comprises proteins with high immunogenicity property (Rohousova et al., 2005; Vlkova et al., 2011). The

detection of this immune response could be useful in an epidemiological setting as marker of sand fly exposure. Differences in the detection of exposure between endemic regions could be due to the sand fly density pattens influenced by temperature, latitude, elevation, season and annual pattern among others.

Detection of anti-sand fly saliva antibodies in canine samples are mainly based on ELISA techniques that use different types of antigens, including the salivary gland homogenate (SGH), which is considered as the gold standard (Burnham et al., 2020), or other recombinant protein such as yellow-related protein rSP03B (Kostalova et al., 2015). Both type of antibodies recognizing SGH and rSP03B are associated to sand fly abundance and it is characterized by a seasonal dynamics of the *P. perniciosus*: increasing anti-salivary antibodies during summer time and decreasing during winter months when sand flies are not active (Kostalova et al., 2015). Further studies are needed to investigate the immunological properties of the antibodies detected in ferrets as these would have important implications for seroprevalence studies.

In conclusion, the results observed in ferrets suggest a variation of anti-*Leishmania* antibodies between the transmission period and no transmission period as previously observed in dogs. Further studies are needed to expand the knowledge about sand fly exposure in other animals such domestic ferrets, detecting the presence of anti-saliva antibodies against *P. perniciosus* and their correlation with other parameters including clinicopathological and immunological information.

CRediT author statement

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Conflict of interest statement

The authors have nothing to disclose.

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Tables

Table 1. OD variation detected by ELISA during the sand fly transmission period, and follow-up during the sand fly non-transmission period.

Animal	OD during transmission period (classification)	OD during non-transmission period (classification)	Follow-up variation
1	0.260 (low)	0.273 (low)	Stable OD

2	0.247 (low)	0.196 (negative)	Reduced OD
3	0.276 (low)	0.239 (low)	Reduced OD
4	0.336 (low)	0.471(medium)	Increased OD
5	0.220 (low)	0.227 (low)	Stable OD
6	0.343 (low)	0.345 (low)	Stable OD
7	0.329 (low)	0.169 (negative)	Reduced OD
8	0.346 (low)	0.430 (medium)	Increased OD
9	0.397 (low)	0.145 (negative)	Reduced OD
10	0.256 (low)	0.190 (negative)	Reduced OD
11	0.202 (low)	0.319 (low)	Increased OD
12	0.731 (high)	0.375 (low)	Reduced OD
13	0.274 (low)	0.239 (low)	Reduced OD
14	0.318 (low)	0.153 (negative)	Reduced OD
15	0.463 (medium)	0.288 (low)	Reduced OD
16	0.382 (low)	0.242 (low)	Reduced OD
17	0.231 (low)	0.215 (low)	Stable OD
18	0.278 (low)	0.326 (low)	Increased OD
19	0.421 (medium)	0.426 (medium)	Stable OD
20	0.417 (medium)	0.174 (negative)	Reduced OD
21	0.227 (low)	0.173 (negative)	Reduced OD