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Short Communication

First report on natural infection with *Leishmania infantum* in a domestic ferret (*Mustela putorius furo*) in Spain

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ABSTRACT

A pet domestic ferret (*Mustela putorius furo*) with a papular lesion involving the right pinna was diagnosed with chronic pyogranulomatous dermatitis by histopathologic examination. Intralesional, intracytoplasmic oval microorganisms compatible with *Leishmania* spp. or *Histoplasma* spp. were observed in macrophages and multinucleate giant cells. *Leishmania infantum* (*L. infantum*) infection was diagnosed by PCR, culture in Novy-MacNeal-Nicolle medium, and immunohistochemistry. Abnormal clinicopathological results included increased alanine transferase, alkaline phosphatase, serum gamma glutamyl transferase and polyclonal gammpathy. Anti-*Leishmania* antibodies were detected by enzyme-linked immunosorbent assay, immunofluorescence antibody test and western blot using *L. infantum* antigen. Immunoreactivity against the 16 kDa specific *L. infantum* antigen fraction was observed by western blot. PCR performed in blood samples obtained from this patient after positive parasite isolation detected *L. infantum* DNA. To the authors' knowledge, this is the first diagnosis and isolation of *L. infantum* in a domestic ferret naturally infected in an endemic region (Spain) where canine and feline leishmaniosis is frequently detected. According to these findings, ferrets should be included as potential reservoir hosts of *L. infantum*. Future investigations should analyze the epidemiological role of ferrets in *L. infantum* infection including the prevalence of infection.

1. Introduction

Leishmaniasis currently ranks second only to malaria in the World Health Organization (WHO) list of protozoan diseases that cause the highest human mortality. It is estimated that approximately 350 million people are under the risk of leishmaniasis infection and 12 million are infected (Alvar et al., 2012). In southern Europe, leishmaniasis is a zoonotic protozoan disease caused by *Leishmania infantum*. This parasite is transmitted by phlebotomine sand flies under natural conditions during feeding. Although various phlebotomine species have been implicated in the transmission of *L. infantum* in Europe, only two species are the most important vector of canine leishmaniosis in the Iberian Peninsula: *Phlebotomus perniciosus* and *Phlebotomus ariasi* (Lucientes et al., 2005). Female *Phlebotomus* feed on a variety of vertebrate hosts, including humans, livestock species, dogs, wild rabbits, hares, rodents and cats.

Dogs are considered the main reservoir host for *L. infantum* but other animals can act as active reservoirs such as cats (Pennisi et al., 2015) and rabbits (García et al., 2014). The identification of other domestic animals that can act as possible reservoir hosts for the parasite could have a significant impact on public health. Equally, from a veterinary point of view, the identification of this disease in a new animal

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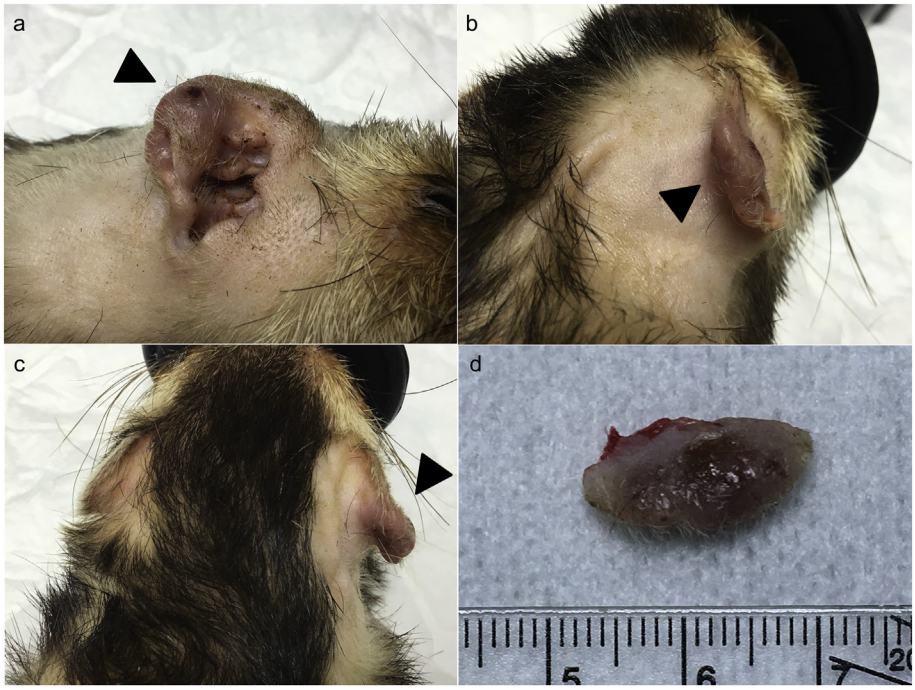

Fig. 1. Different views of dermatologic lesions detected in this ferret (a, b, c) and full thickness biopsy performed from the ear pinna (d).
a, b, c: Erythematous and oedematous papular lesion on the right ear pinna (arrowheads). A superficial ulcer is present on the tip of the ear pinna (a, b).

species is deemed essential.

The potential role of wildlife species as reservoirs of the parasite should be taken into account as well. Among mustelids, *L. infantum* infection has been identified by PCR in liver and/or spleen tissue samples from a polecat (*Mustela putorius*), a European mink (*Mustela lutreola*) and other wild mustelids (*Meles meles, Martes foina* and *Martes martes*) in the northern Iberian Peninsula (Spain) (Del Río et al., 2014). None of the animals showed typical canine leishmaniosis lesions at post-mortem examination.

Although no official ferret population data are available in Europe (d'Ovidio et al., 2014), the domestic ferret (*Mustela putorius furo*) is nowadays a common household pet. This study describes for the first time naturally-occurring *L. infantum* infection in a ferret.

2. Case report

A 4-year-old intact female ferret from Valencia, in the east coast of Spain, was clinically evaluated in February 2019 because of the presence of a non-pruritic dermal lesion in the right pinna (Fig. 1). This ferret was adopted at the age of two years with unknown previous history. At the time of presentation, it lived in an apartment with other ferrets and had access to an outdoor terrace. It was under chronic medical management with prednisolone and cyclosporine A because of inflammatory bowel disease diagnosed one year earlier. This patient was also diagnosed with suppurative cholangitis six months prior to dermatologic presentation.

On physical examination, this ferret was in good condition, active and alert, normothermic and properly hydrated. Cardiac auscultation was within normal limits. Respiratory sounds were also normal and there was no evidence of lymph node enlargement. The fur over the back was coarse. An erythematous, edematous and non-painful papular lesion 5 mm in diameter was observed in the right ear pinna. Cytological evaluation from a sample taken by needle aspiration and stained with Diff-Quick revealed pyogranulomatous dermatitis; no infectious agents were visualized. A full thickness biopsy from the ear pinna was obtained.

2.1. Histopathology and immunohistochemistry specific against L. infantum

The skin biopsy sample was fixed in 10% formalin and embedded in paraffin. Four µm-thick sections were stained with hematoxylin and eosin. Severe chronic diffuse pyogranulomatous dermatitis extending diffusely from the superficial dermis to the subcutis was noted. Inflammation was characterized by dense infiltrates of macrophages, few neutrophils, low numbers of lymphocytes and plasma cells, and rare multinucleate giant cells. The auricular cartilage was unaffected. This lesion was accompanied by areas of necrosis in the dermis and proteinaceous edema and serocellular crusts. Macrophages and multinucleate giant cells contained large numbers of oval organisms with an eccentric nucleus and pale cytoplasm, measuring approximately 3 to 4 µm in diameter, compatible with *Leishmania* spp. amastigotes or *Histoplasma* spp. yeasts were observed (Fig. 2). To determine the presence of *Leishmania* parasites in tissue section, immunohistochemistry was performed using a standard protocol with an Autostainer Link48™ (Dako, Glostrup, Denmark) and an in-house rabbit polyclonal antibody specific for *L. infantum*. Blocking of endogenous peroxidase activity (Dako REAL™ Peroxidase-Blocking Solution, Dako, Glostrup, Denmark) was performed before sections were incubated for 30 min with the primary antiserum at room temperature (RT) (1 in 500 dilution in EnVision Flex™ antibody diluent, Dako). Thereafter, sections were incubated for 30 min at RT with Dako EnVision+ System-HRP,Rb™. The substrate used for detection was 3.3′-diaminobenzidine incubated for 10 min. Sections were then counterstained with hematoxylin for 8 min (EnVision™ FLEX Hematoxylin, Dako) and covered in slides. For the negative control, the primary antibody was replaced with non-immune rabbit serum. A biopsy sample of the lymph node from a dog with clinical leishmaniosis was included as a positive control. The abundant oval microorganisms present within the cytoplasm of dermal macrophages stained positively (brown) (Fig. 3).

2.2. PCR

The presence of *Leishmania* spp. DNA in paraffin embedded skin biopsy was additionally evaluated by amplification of kinetoplast DNA

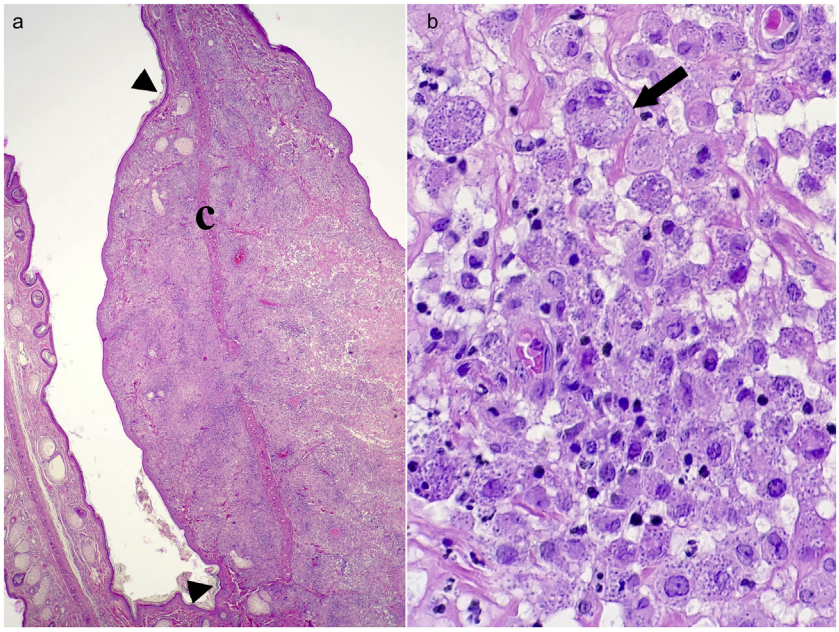

Fig. 2. Histological sections of affected tissues with leishmaniosis in a ferret.
a: Note a papular thickening of the ear (delimited with arrowheads) due to granulomatous inflammation. c = auricular cartilage.
b: A higher magnification of this inflammatory lesion reveals infiltrates of macrophages and low numbers of lymphocytes as a well as a multinucleate giant cell (arrow). The cytoplasm of macrophages and this giant cell are ladden with oval microorganisms with an eccentric nucleus. Hematoxylin and eosin.

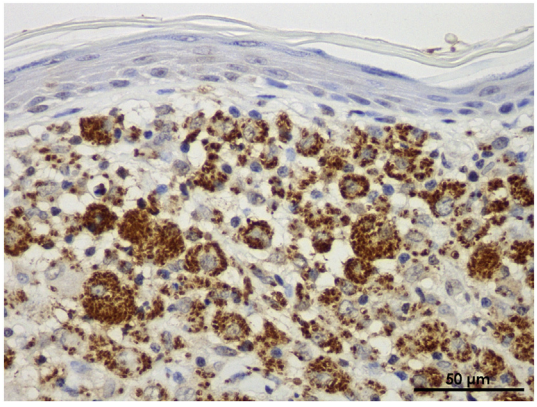

Fig. 3. Immunohistochemical staining labelling of *Leishmania* spp. amastigotes. Note the amastigote forms labelled in brown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sequence using a quantitative polymerase chain reaction (qPCR). Each amplification was performed in triplicate, in 10 µL reaction, 15 pmol of direct primer (5′-CTT TTC TGG TCC TCC GGG TAG G-3′), 15 pmol of reverse primer (5′-CCA CCC GGC CCT ATT TTA CAC CAA-3′), 50 pmol of the labelled TaqMan probe (FAM-TTT TCG CAG AAC GCC CCT ACC CGC-TAMRA) and 2.5 µL of sample DNA. Amplification and detection were performed in the ABI Prism 7900 system (Applied Biosystems, Foster City, CA, USA.) in a two-step temperature process (94 and 55 °C) for 45 cycles. Positive controls (DNA from *L. infantum* MHOM /ES /04 /BCN-61) and negative controls were included in each RT-PCR analysis (Martín-Ezquerra et al., 2009). A positive result by PCR was obtained from the paraffin block. Additional diagnostic procedures included detection of parasite DNA by PCR from peripheral blood sample and Whatman filter paper number 3 with aspirated material from the perilesional excised area, serology and parasite isolation. Concerning the blood sample and Whatman filter paper results, *Leishmania* spp. DNA was detected in both samples.

2.3. Serology

Anti-*Leishmania* antibodies were detected by western blot (WB) and enzyme-linked immunosorbent assay (ELISA) using sonicated *L. infantum* antigens (MHOM/FR/78/LEM75 zymodeme MON-1). WB was performed as described by Riera et al. (1999), with some modifications. It was done on 0.1% SDS–13% polyacrylamide gel on a Mini-gel Bio RadSystem. Sera diluted at 1/50 were assayed and a protein A peroxidase conjugate (1/1000 dilution; Pierce) was used. A serum positive was considered when immunoreactivity against the 14 and/or16 kDa *Leishmania* antigen fraction was observed. ELISA was performed as described by Riera et al. (1999). Sera were diluted at 1/50 and a protein A peroxidase conjugate (dilution, 1/8000; Pierce) was used. The cutoff was set to 0.137 optical density units (OD) (mean + 4 standard deviations of values from 8 ferrets with a negative result for *Leishmania* real-time PCR in blood). Each test included serum from a *L. infantum* confirmed symptomatic dog from Spain with a *L. infantum* isolation as positive control and serum from a healthy, non-infected dog as negative control because the protein A peroxidase conjugate has been employed in canine samples. Immunoreactivity from the *Leishmania* antigen was detected by WB (band of 16 kDa) and specific antibodies against *L. infantum* were detected by ELISA. Medium levels of antibodies were determined with an OD result of 0.599.

Later, other quantitative serological tests were performed including an in-house immunofluorescence antibody test (IFAT), for detecting *L. infantum* immunoglobulin G antibodies using *L. infantum* (strain MHOM/FR/78/LEM75 zymodeme MON-1) as whole-parasite antigen fixed on multi-spot slides (Thermo Fisher Scientific, Waltham, Massachusetts, USA). All samples were diluted 1:20 with phosphate-buffered saline (PBS), and the endpoint titer of positive samples was determined by preparing serial twofold dilutions of the serum starting from the cutoff value. Immediately thereafter, 20 µL of every diluted serum sample were applied per well. The slides were incubated for 30 min at 37 °C in a moist chamber. Slides were then washed twice with PBS for 5 min and once with distilled water. Following the washing procedure, 20 µL of fluorescein-conjugated goat anti-ferret immunoglobulin G (Abcam, Cambridge, United Kingdom) diluted 1:16 in Evans blue solution were added to each well. The slides were incubated in a moist chamber at 37 °C for an additional 30 min in the dark. The washing procedure was repeated, and several drops of mounting medium were then added to the cover slips. Slides were examined under a fluorescence microscope (Leica DM750 RH; Leica Microsystems, Wetzlar, Germany) at 400 × magnification and each well was compared to the fluorescent pattern observed in the positive

and negative controls. The samples were examined by two different researchers. The cutoff for this technique was set to 1:20 dilution by analyzing 8 serum samples from ferrets with a negative result for *Leishmania* real-time PCR in blood. The same procedure was repeated with the modification of fluorescein-conjugated goat anti-dog immunoglobulin G (Abcam, Cambridge, United Kingdom). Due to the lack of ferret positive reference serum samples, the ferret IFAT was determined by carrying out a parallel canine test run, using positive serum control (a dog from Spain with *L. infantum* confirmed with positive *L. infantum* culture) and negative serum control (dog from United Kingdom, a non-endemic area). Positive and negative controls were included on each slide. The result of the ferret IFAT was positive at 1:160 dilution with fluorescein-conjugated goat anti-ferret immunoglobulin G. However, a negative result of the ferret serum sample was obtained with fluorescein-conjugated goat anti-dog immunoglobulin G.

2.4. In vitro isolation and cultivation

For parasite isolation, Novy–MacNeal–Nicolle (NNN) medium and Schneider medium supplied 100 IU/ mL penicillin and 100 μg/mL streptomycin solution and 10% foetal calf serum was used.

Several drops of the aspirated material from the perilesional skin excised area were introduced into the liquid phase of the NNN and were incubated in medium Schneider at 26 ± 1 °C and cultures were microscopically assessed every day. The culture was positive after three days of incubation and *Leishmania* spp. strain isolated (Clinical Immunology Laboratory, University of Zaragoza) was molecularly type as isolated as *L. infantum* (Laboratory of Parasitology, University of Barcelona) and named MMST/ES/2019/ZGZ103.

2.5. Clinicopathological findings

Complete blood cell count revealed hematological values within normal limits and serum biochemistry profile showed elevated serum alanine transferase (ALT: > 1000 U/L, reference 82–289 U/L), alkaline phosphatase (ALKP: 239 U/L, reference 9–84 U/L), serum gamma glutamyl transferase (GGT: 250 U/L reference 0.2–14 UI/L), alpha-2 globulins (11.1 g/L; reference 4.2–8.5 g/L) and gamma-globulins (22 g/L; reference 2.7–9 g/L (4.8–14.7%)) with an A/G ratio of 0.69 (reference 0.58–1.33). Serum protein electrophoresis revealed polyclonal gammopathy.

3. Discussion

To the authors´ knowledge, this report describes the first clinical case of leishmaniosis in a domestic ferret (*Mustela putorius furo*) and is the first notification of natural *L. infantum* infection detected by parasite culture in mustelids. The diagnosis of clinical leishmaniosis in this ferret was based on the clinical signs, pathologic findings, and confirmatory *L. infantum* techniques, particularly PCR and culture. However, experimental infection has been described in ferrets used as animal models for the study of antileishmanial compounds (White et al., 1989). In dogs, the evolution and pathogenesis of natural infection is highly variable and not easily comparable with experimental infection. There may also be differences in the clinical course as well as in antibody production, among others. This situation therefore could be extrapolable in ferrets.

In the past decade wild animals such as lagomorphs and canids other than dogs have been attributed an important role in the transmission cycle of *L. infantum* (Miró et al., 2017a). Domestic ferrets have probably not played a crucial role as reservoir for *L. infantum* infection; however, studies may be necessary to elucidate the epidemiological role of domestic ferrets as household pets in this transmission cycle.

The ferret from this case report was infected in an urban environment in Valencia, on the East coast of the Iberian Peninsula fronting the Gulf of Valencia on the Mediterranean Sea, a geographical area where *P. perniciosus* is the most prevalent phlebotomine species detected (Aransay et al., 2004). Recent data about seropositivity rate of *L. infantum* in dogs in the East of Spain confirms that detection of anti-*Leishmania* antibodies was higher compared to the rest of the pathogens analyzed including *Anaplasma* spp., *Ehrlichia canis*, *Borrelia burgdorferi* and *Dirofilaria immitis* in this region (Miró et al., 2013). Human leishmaniasis has also been detected in the Valencia region and a recent outbreak of cutaneous leishmaniasis was described (Roth-Damas et al., 2017). In this sense, One Health context confirms the endemic nature and the high prevalence of the infection in the European Mediterranean regions and the potential possibility to detect the presence of *L. infantum* infection in other animals living in the same geographical area.

The presence of parasite in blood samples should be considered an important factor in the risk of iatrogenic transmission of *L. infantum* by blood transfusion as it is reported in other species such as dogs (Tabar et al., 2008). Moreover, transmission by vectors should be taken into account and preventive measures to avoid infection transmission from this and any other ferrets should be applied as is done in dogs (Miró et al., 2017b) and cats (Brianti et al., 2017).

A broad range of immune responses and clinical manifestations have been described in canine leishmaniosis. Infection in dogs may be subclinical or manifested as a self-limiting disease, or a severe, and sometimes, fatal illness. Subclinical infection is not necessarily permanent and factors such as immunosuppression or concomitant diseases could break the equilibrium and lead to the progression of clinical disease in dogs (Solano-Gallego et al., 2009) as in cats (Pennisi et al., 2015) and humans (Alvar et al., 2012). Although ferrets are often relatively resistant to the immunosuppressive effects of prednisolone, this patient was under immune-modulating drugs therapy (prednisolone and cyclosporine) that might have caused immunosuppression and contributed to the clinical manifestation of *Leishmania* infection.

The macroscopic skin lesions of canine and feline leishmaniosis can be diverse, even in the same patient. The main clinical presentations include exfoliative, ulcerative, nodular, sterile pustular and papular dermatitis, nodules at the site of parasite inoculation and onychogryphosis (Manolis et al., 2014; Pennisi et al., 2015). These lesions are the result of granulomatous or pyogranulomatous inflammation that targets different structures of the skin and of the deposition of immune complexes (Manolis et al., 2014). The ferret of this report presented with pyogranulomatous dermatitis compatible with a less common clinicopathological presentation of the disease (papular dermatitis or nodule at the site of parasite inoculation). In dogs, each presentation may reflect a different host–parasite relationship, as in the case of the nodular and papular dermatitis, which are considered markers of high and low susceptibility to canine leishmaniosis, respectively (Manolis et al., 2014).

Clinicopathological findings detected in this ferret included moderately increased serum enzyme activities (ALT, ALKP and GGT) compatible with liver disorders and polyclonal hypergammaglobulinemia. Inflammatory, infectious and toxic hepatic disease. Hepatic lipidosis, and hepatic neoplasia are the most common hepatic diseases encountered in this species (Huynh and Laloi, 2013). Hepatic disease often remains subclinical, which may lead to difficulties in diagnosis. Canine leishmaniosis can occasionally cause hepatitis and elevation of liver enzymes (Rallis et al., 2005). In the ferret of this report, the cause of ALT, ALKP and GGT elevations and if these were related to leishmaniosis cannot be determined because liver biopsies were not obtained.

Hypergammaglobulinemia in ferrets is associated with a variety of infections including Aleutian disease, systemic coronavirus, canine distemper virus, certain neoplasia or systemic mycoses (e. g., blastomycosis and coccidioidomycosis) (Melillo, 2013). In canine leishmaniosis, serum protein electrophoresis reveals an increase of total proteins and globulins, as well as a typically polyclonal gammapathy. In this regard, the serum electrophoretogram detected in the ferret was

very similar to the typical serum protein electrophoresis in dogs (Maia and Campino, 2018) and cats (Pennisi et al., 2013) with clinical leishmaniosis, but not specific for a single infectious disease in ferrets.

The diagnosis of canine and feline leishmaniosis traditionally focuses on the detection of specific antibodies against *L. infantum* in clinical and research settings, with numerous serological techniques developed including IFAT, ELISA and WB techniques. The validation and adaptation of each serological technique to the animal species and reagents employed to perform the technique requires the use of antisera specific to the animal species analyzed. In this regard, serological evaluation of this ferret was performed with two different anti-immunoglobulin G conjugates in the in-house IFAT technique, with a positive result only with specific detection of anti-ferret immunoglobulin G instead of anti-dog immunoglobulin G. Protein A as a conjugated reagent, by contrast, is capable of interacting with the fragment crystallizable region from immunoglobulin G of several animals including ferret, dog (Riera et al., 1999) and cat (Solano-Gallego et al., 2007), being this reagent is very useful to determine total serum immunoglobulin G in animals with clinical leishmaniosis at the time of diagnosis.

The results described are strongly suggestive of a possible role of ferrets as reservoir of *Leishmania* infection. Further epidemiological studies are needed to understand the role of ferrets as a potential reservoir for human infection and how the infection could develop into the disease over time. Equally, therapeutic management for anti-*Leishmania* treatment in pet ferrets should be considered.

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Declaration of Competing Interest

The authors have nothing to disclose.

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