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SHORT COMMUNICATION

First serological study of *Dirofilaria immitis* antibodies in household domestic ferrets (*Mustela putorius furo*) in southern Spain – Short communication

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ABSTRACT

Dirofilaria immitis is an endemic mosquito-borne pathogen widely spread throughout Europe as well as North and South America. Infection by *D. immitis* has been reported in domestic ferrets, although little is known about the occurrence and the epidemiological features of this nematode in this species. The aim of the present retrospective study was to assess the prevalence of *D. immitis* antibodies using an in-house enzyme-linked immunosorbent assay specifically developed for use in ferrets. One hundred and eighty-six serum samples were obtained from the Province of Valencia (Spain), an area endemic for dirofilariosis. Of the 186 serum samples included in the study, 27 (14.51%) were classified as *D. immitis* seropositive and 159 samples as *D. immitis* seronegative. The results provide valuable information on the seroprevalence of *D. immitis* infection in domestic ferrets in an area endemic for this vector-borne pathogen. The presence of seropositive ferrets should be taken into account and preventive measures should be implemented, including the possibility of serological screening for the early detection of *Dirofilaria* antibodies as a serological marker of exposure. This is the first study that demonstrates the presence of *D. immitis* exposure in ferrets in Spain. Veterinarians working in endemic areas should be aware of this infection in ferrets and their susceptibility.

KEYWORDS

dirofilariosis, heartworm disease, ferret, *Mustela putorius furo*, serology, Spain

Filarial worms are vector-borne nematodes that infect mainly dogs but also cats, ferrets, wild carnivores (foxes, jackals, coyotes, wolves, raccoons, wild felids, sea lions, black bears) and humans (Pennisi et al., 2020). Dirofilariosis is a globally spread heartworm disease caused by *Dirofilaria immitis* and transmitted by culicid mosquitoes during blood feeding under natural conditions (McCall et al., 2008). Dogs are considered the main reservoir host of *D. immitis* (Simón et al., 2012) and host–parasite relationships between *D. immitis* and domestic dogs and cats have been extensively studied, but there have been relatively few reports on infections in ferrets (Sasai et al., 2000; Bradbury et al., 2010; Molnár et al., 2010).

Nowadays the domestic ferret (*Mustela putorius furo*) is a common household pet across Europe and the United States of America. It is known that ferrets are highly sensitive to *D. immitis* and that the parasite can complete its life cycle in this species (Sasai et al., 2000). The susceptibility and life cycle of this parasite have been studied in this species and they are

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similar to those of heartworm in dogs; however, because of the small size of ferrets, the clinical presentation resembles that of infected cats (Morrisey and Malakoff, 2021).

The diagnosis of *Dirofilaria* infection is complicated and depends on several factors such as host species, site preference, infection status, sex, and parasite load (Laidoudi et al., 2021). Heartworm-infected ferrets have low, transient concentrations of microfilariae, making microfilaria detection tests unreliable. However, the detection of microfilariae provides definitive evidence of infection (McCall, 1998). Enzyme-linked immunosorbent assay (ELISA) based antigen tests have been shown to be effective 5–6 months after infection, but may show false negative results due to low worm burdens (Wagner, 2009). Furthermore, the antigen tests detect only antigens shed into the circulation by adult female heartworms (Morrisey and Malakoff, 2021). Thus, the results of antigen tests detecting glycoproteins secreted by female heartworms will be false negative in the case of a male-only infection (Kondert and Mayer, 2018). Diagnostic approaches available for ferret dirofilariosis include molecular assays specific for *D. immitis* and echocardiography. However, the small body size of ferrets makes the detection of adult heartworms difficult. By echocardiography, parasites sometimes can be seen in the right heart chambers, the pulmonary artery or the distal caudal vena cava (Wagner, 2009). Due to the limitations of these diagnostic tools, the best current practice suggests a combination of techniques to confirm heartworm disease in ferrets. The combination of heartworm antigen test and imaging techniques (thoracic radiographs, echocardiography, and angiography to detect adult heartworms in the heart and associated vessels) appears to yield a relatively high and accurate detection rate (Zaffarano, 2015).

Although several case reports of heartworm disease in ferrets have been published, information about the geographical distribution and epidemiological features of *D. immitis* infection in ferrets is scarce, and the prevalence of natural dirofilariosis in the domestic ferret is unknown so far. Clinical signs associated with the presence of the nematode in affected ferrets include coughing, lethargy, weakness, dyspnoea, and hypothermia. Echocardiographic examination may identify the presence of parasites in the pulmonary artery, right ventricle, or right atrium (Morrisey and Malakoff, 2021). Similarly, the presence of pulmonary hypertension should also be suspected that can be diagnosed by the use of Doppler echocardiography. The detection of microfilaraemia is a traditional diagnostic tool for dirofilariosis (Zaffarano, 2015). However, the short-term occurrence of microfilaraemia and the small amount of microfilariae due to the often low number of adult female worms make it difficult to diagnose *D. immitis* infection in ferrets (Wagner, 2009; Morrisey and Malakoff, 2021), compared to dogs. The combined use of DNA-based PCR assays and commercial antibody tests could increase the diagnostic accuracy of the detection of *D. immitis* infection in this species, as well as in cats (Pennisi et al., 2020). Other methods including the detection of *D. immitis* antibodies by an in-house ELISA using somatic antigens from third-stage larvae of *D. immitis* (SL3) could be useful as an early diagnostic marker of heartworm infection in this species (Prieto et al., 2001).

In an epidemiological study done on cats, the prevalence detected using antigen tests was significantly lower than the actual amount of animals having anti-*D. immitis* antibodies (Villanueva-Saz et al., 2021). A positive serological result is indicative of the exposure of the cat's immune system to the parasite; however, it not does not indicate whether it is a previous or a current infection. Cats have a natural resistance to the parasite (Montoya-Alonso et al., 2022), so for many infections, the parasite is likely to be neutralised by the feline immune system, although the antibodies can remain present for an indefinite period of time. Nevertheless, a seropositive cat has undoubtedly been exposed to the parasite, therefore cats could be at risk of infection in general (Montoya-Alonso et al., 2022).

The purpose of this retrospective study was to determine the seroprevalence of *D. immitis* in domestic ferrets by detecting *D. immitis* antibodies using an in-house ELISA assay developed in the present study.

Residual serum samples of client-owned ferrets were obtained from a total of 186 patients seen for medical reasons or routine healthcare check-ups at the Menescalia Veterinary Center in Valencia, in the Province of Valencia on the east coast of the Iberian Peninsula (39°28′12.864″ N, 0°22′36.48″ W), which is an area endemic for heartworm disease. Serum samples were collected aseptically by venipuncture of the cranial vena cava with the owner's consent during the period from January 2020 to March 2021 and blood samples were stored at −20 °C until processing. A complete physical examination was carried out before sampling. Data on age, sex, lifestyle (indoor, outdoor and mixed), cohabitation with dogs as well as clinical information including heart disease or respiratory disease were recorded.

This study required official ethical approval which was granted by the Ethics Committee of the University of Zaragoza (protocol code PI25/20). The animals were handled according to the appropriate ethical standards and the national legislation. In addition, owners were asked to sign a consent to allow the use of samples for research purposes such as this study.

The ELISA was performed on all sera as described previously, with some modifications (Villanueva-Saz et al., 2021). A 100-µL aliquot of ferret sera, diluted 1:100 in phosphate-buffered saline (PBS), was added to each well. The plates were then incubated at room temperature (22–25 °C) in a moist chamber for 45 min, then they were washed with PBS containing 0.05% Tween 20 (PBST) and 100 µL of Protein A conjugated to horseradish peroxidase (Reference: 32,400, Thermo Fisher Scientific) diluted 1:10,000 in PBST, and 1% dry skimmed milk was added. This conjugate was previously used for serology to detect other pathogens such as *Leishmania infantum* in different species including dog (Villanueva-Saz et al., 2022), cat (Alcover et al., 2021) and mustelids such as ferret (Giner et al., 2020) and mink (Giner et al., 2022). The plates were incubated in the moist chamber at 37 °C for 30 min and were washed again with PBST as

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described above. The substrate solution (ortho-phenylenediamine) and stable peroxide substrate buffer (Thermo Fisher Scientific) were added to each well and the reaction was allowed to develop in the dark at room temperature for 20 ± 5 min. The reaction was stopped by adding 2.5 M H_2SO_4 to each well. Absorbance values were read at 492 nm in an automatic ELISA reader (ELISA Reader Labsystems Multiskan, Midland, ON, Canada). As a positive control, each plate included serum from a cat infected by *D. immitis* from an experimental study, and as a negative control, serum from a healthy, non-infected cat. The cut-off was established at optical density (OD) 0.31 (0.29 ± 0.02) (based on the mean OD ± standard deviation detected in 70 non-infected, indoor ferrets from a non-endemic area; these samples were not included in this study as they originate from outside the study area) and thus an OD > 0.31 was considered positive. The in-house ELISA was validated using 12 feline sera positive for *D. immitis* and provided by TRS Labs (GA, USA). These samples contained a known but variable number of female and/or male worms. Moreover, they were evaluated by three different commercially available tests, including two antigen tests: Uranotest *Dirofilaria*® (Urano Vet SL, Barcelona, Spain) and Filarcheck® (Agrolabo Spa, Scarmagno, Italy) and one antibody test (Solo Step® FH), with all giving a positive result. These tests were performed in a private laboratory (I + D, Spain).

Data collected from the entire sample set were analysed using descriptive statistics (Fisher's exact test or chi-square test). Correlations between the presence of anti-*D. immitis* antibodies and the recorded variables were analysed (age, sex, lifestyle, cohabitation with a dog, signs compatible with heart disease or respiratory disease). The significance of differences was assessed using Fisher's exact test/chi-square test. The difference was considered significant at $P \leq 0.05$. The SPSS program (SPSS Inc., Chicago, USA) was used for statistical analyses.

A total of 186 serum samples were analysed from 98 male and 88 female ferrets in this retrospective study (Table 1). All the examined ferrets had a mixture of coat colours and no ferrets had been surgically neutered. The age of the ferrets ranged from 1 to 9 years. The average age of the animals was 4 years, and they were classified as young (<2 years), adult (from ≥2 years to ≤6 years) and senior (>6 years). None of the ferrets had been treated with a long-acting topical antiparasitic repellent against sandflies. The overall seroprevalence of dirofilariosis was 14.51% (95% Confidence Interval 0.10–0.20) by the in-house ELISA (Table 1). No significant correlations were found between positivity for anti-*D. immitis* antibodies and age, sex, lifestyle or cohabitation with dogs ($P > 0.05$). From a total of 27 seropositive ferrets, 6 animals showed clinical signs of heart disease. One of them (1/6) presented an asymptomatic atrioventricular block grade 2 diagnosed by electrocardiography and echocardiographic examination, and three animals (3/6) had heart murmur and pulmonary oedema associated with a mitral valve insufficiency, detected by radiographic and echocardiographic examination. Two ferrets (2/6) had heart murmur without pulmonary signs, and mitral valve insufficiency was detected by echocardiographic examination. Abnormal hyperechoic structures in the right atrium, in the right ventricle or in the cranial vena cava were detected. Moreover, significant correlation was found between *D. immitis* seropositivity and the presence of clinical signs associated with heart disease ($P = 0.02$), respiratory disease ($P = 0.03$), and the presence of clinical signs linked to heart or respiratory disease ($P = 0.008$).

To the best of the authors' knowledge, the present study is the first report of *D. immitis* antibody detection in domestic ferrets in Spain. The information existing on the distribution of *D. immitis* and epidemiology of heartworm disease in domestic ferrets in an endemic area of the Iberian Peninsula is rather incomplete. Regarding canine dirofilariosis, a recent study revealed that its prevalence is 6.95% in dogs in the Valencian region, based on the results of immunochromatographic tests of *D. immitis* antigens (Montoya-Alonso et al., 2020).

The clinical features of feline heartworm disease make it difficult to diagnose the disease in cats and also in domestic

Table 1. Serological results of *Dirofilaria immitis* infection in the ferrets examined and its correlation to various factors

Examined factors		*Dirofilaria*-specific antibodies by ELISA		
		No. of positive ferrets/total no. of ferrets examined	Positive result (%)	Significant difference detected ($P < 0.050$)
Sex	Female	14/88	15.91	No ($P = 0.679$)
	Male	13/98	13.27	
Age	Young (<2 years)	2/41	4.88	No ($P = 0.052$)
	Adult (2–6 years)	22/127	17.32	
	Senior (>6 years)	3/18	16.67	
Habitat	Indoor	16/93	17.20	No ($P = 0.911$)
	Outdoor	2/22	9.09	
	Mixed	9/71	12.68	
Cohabitation with a dog	No	20/153	13.07	No ($P = 0.284$)
	Yes	7/33	21.21	
Clinical signs associated with heart or respiratory disease	Yes	6/6	100	Yes ($P = 0.008$)
	No	21/180	11.67	
Seroprevalence in total		*27/186*	*14.51*	*Not available*

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ferrets; therefore, epidemiological data referring to cats or ferrets are rather scarce. Recent studies on feline dirofilariosis carried out in Barcelona and Zaragoza detected 14.47% and 25.20% *D. immitis* seroprevalence, respectively (Montoya-Alonso et al., 2014; Villanueva-Saz et al., 2021). The present study focused on *D. immitis* infection in domestic ferrets, and detected similarly high, 14.51% seroprevalence in household domestic ferrets from the Province of Valencia, on the east coast of the Iberian Peninsula.

PCR assays are capable of sensitive and specific identification of the *D. immitis* genetic material in blood. Theoretically, a single heartworm cell can be detected, making PCR a useful tool for the early detection of heartworm infection (Wagner, 2009). In contrast, serological tests for *Dirofilaria* are indirect fluorescent antibody tests, and generally detect antibody against microfilaria or adult *Dirofilaria*, whereas ELISA tests detect specific antibody or antigen. The use of serological tests that detect IgG response to heartworm infection is available for the early detection of *Dirofilaria* antibodies as a serological marker in cats (American Heartworm Society, 2014). In this sense, the presence of anti-*D. immitis* antibodies in other animals different from dogs, such as cats (Montoya-Alonso et al., 2022) or ferrets, highlight the epidemiological importance of infection, especially in areas where heartworm disease is endemic. Thus, for a better understanding of the epidemiology of this infection in ferrets, it is necessary to examine the development of this parasitosis in seropositive ferrets to decide on the adequate diagnostic approach.

No significant correlation was found between positivity for anti-*D. immitis* antibodies and the sex of ferrets. Similarly, we did not find a correlation between infection prevalence and the lifestyle of ferrets. Although dirofilariosis is transmitted by infected mosquitos, the seropositivity was notably higher in the indoor ferrets (16/93; 17.20%) than in the outdoor ones (2/22; 9.09%). The examined ferrets living both indoors and outdoors had a seropositivity rate of 12.68% (9/71). A possible explanation for the high seropositivity in indoor ferrets compared to those with an outdoor lifestyle is the fact that building constructions are able to create environments (vegetation and artificial water sources) that facilitate the proliferation of vectors, some of them with microfilaria. Moreover, it was proven that cats with an indoor lifestyle were not protected from infection (Montoya-Alonso et al., 2014).

The results of this study show the need of implementing preventive measures including chemoprophylaxis against mosquitoes throughout the transmission period, to avoid their interaction with ferrets and the parasite in endemic regions. Other possible measures to avoid mosquito bites is the use of registered repellents against mosquitoes. In domestic pet ferrets, the use of this type of products is off-label and the application of pyrethrin and pyrethroids labelled for dogs may cause neurological signs (Dunayer, 2008). For this reason, it would be advisable to study and evaluate repellent compounds in this species to have a suitable alternative.

The antibody seropositivity does not show an actual prevalence of infection, thus it is an indirect way to determine the current risk of heartworm infection. Nevertheless, the present study revealed high *D. immitis*-seropositivity in household ferrets in a *D. immitis*-endemic area of Spain, and suggests that further epidemiological studies using serological diagnostic tools are needed to understand the role of ferrets as a potential reservoir of *Dirofilaria* infection.

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