Short communication

First serological study of *Dirofilaria immitis* antibodies in household domestic ferrets (*Mustela putorius furo*) in the Southern of Spain

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Abstract:

Dirofilaria immitis is an endemic mosquito-borne pathogen widely spread throughout Europe, North and South America. *D. immitis* infection has been reported in domestic ferrets although little is known about the occurrence and the epidemiological information of this pathogen in this species. The present retrospective study is aimed at assessing the prevalence of *D. immitis* antibodies using an in-house enzyme-linked immunosorbent assay specifically developed to use in ferrets. A hundred and eighty-six serum samples were obtained from the Province of Valencia (Spain), a dirofilariosis endemic area. Of the 186 serum samples included, 27 (14.51%) were classified as *D. immitis* seropositive and 159 samples were classified as *D. immitis* seronegative. The results obtained provided valuable information regarding the seroprevalence of *D. immitis* infection in domestic ferrets in an endemic area 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 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

Filarioid 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)

The domestic ferret (*Mustela putorius furo*) is a common household pet across Europe and the United States of America nowadays. 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 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).

Dirofilaria infection diagnosis 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 microfilaria, making microfilaria detection tests unreliable. However, 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 to 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, 2018). Molecular assays specific for *D. immitis* and echocardiography are available diagnostic approaches of ferret dirofilariosis. 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, pulmonary artery or 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 to the presence of the nematode in affected ferrets include coughing, lethargy, weakness, dyspnea, 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 and can be diagnosed with the use of Doppler echocardiography. The detection of microfilaremia 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 (Morrisey and Malakoff, 2021; Wagner, 2009), compared to dogs. The combined use of

DNA-based PCR assays and the commercial antibody tests could increase the diagnostic accuracy for the detection of *D. immitis* infection in this species, as well as for 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 reported, the prevalence detected using antigens tests can be significantly lower than the actual amount of animals with anti-*Dirofilaria immitis* antibodies (Villanueva-Saz et al., 2021). A positive result by serology is indicative of exposure of the cat's immune system to the parasite, however, not indicative of whether the infection is previous or present. Cats have a natural resistance against the parasite (Montoya-Alonso et al., 2022), so for many infections, the parasite is likely to be neutralized by the feline immune system, although the presence of antibodies can remain 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, 2022).

The purpose of this retrospective study was to determine the *D. immitis* seroprevalence of 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 one hundred and eighty-six patients seen for medical reasons or routine healthcare check-ups at 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) that is an endemic area of heartworm disease. Serum samples were collected aseptically by cranial cava venipuncture 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, gender, lifestyle (indoor, outdoor and mix), cohabitation with dogs as well as clinical information including heart disease or respiratory disease were recorded.

This study required official ethical approval being conducted according to 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 buffer 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: 32400, 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 pathogen 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 described above. The substrate solution (ortho-phenylene-diamine) 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

137 included serum from a cat infected by *D. immitis* from an experimental study, and as a
138 negative control, serum from a healthy, non-infected cat. The cut-off was established at
139 optical density (OD) 0.31 (0.29±0.02) (based on the mean OD + 4 standard deviation
140 detected in 70 non-infected, indoor ferrets from non-endemic area; these samples were
141 not included in this study as they originate from outside the study area) and thus OD>0.31
142 were considered positive. The in-house ELISA was validated using 12 feline sera positive
143 for *D. immitis* and provided by TRS Labs (GA, USA). These samples contained known
144 but variable number of female and/or male worms. Moreover, they were evaluated for
145 three different commercially available tests including two antigen tests: Uranotest
146 *Dirofilaria*® (Urano Vet SL, Barcelona, Spain) and Filarcheck® (Agrolabo Spa,
147 Scarmagno, Italy) and one antibody test (Solo Step® FH) with all giving positive result.
148 These tests were performed in a private laboratory (I+D, Spain).

149 Data collected from the entire sample set were analyzed using descriptive statistics
150 (Fisher´s exact test or chi-square). Correlations between the presence of anti-*Dirofilaria*
151 *immitis* antibodies and the recorded variables were analyzed (age, gender, lifestyle,
152 cohabitation with dog, compatible signs with heart disease or respiratory disease). The
153 significance of differences was assessed using Fisher´s exact test/chi-square. The
154 difference was considered significant at $p \leq 0.05$. The SPSS program (SPSS Inc.,
155 Chicago, USA) was used for statistical analyses.

156 A total of 186 serum samples were analyzed from 98 male and 88 female ferrets in this
157 retrospective study (Table 1). All the examined ferrets had a mixture of coat colours and
158 no ferrets had been surgically neutered. The age of the ferrets ranged from 1 to 9 years
159 old. The average age of the animals was 4 years, and they were classified as young (<2
160 years), adult (from ≥2 years to ≤6 years) and senior (>6 years). None of the ferrets had
161 been treated with a long-acting topical anti-parasitic repellent against sand flies. 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, gender, lifestyle or cohabitation with dogs ($p > 0.05$). From a total of 27 seropositive ferrets, 6 animals presented 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) presented heart murmur and pulmonary edema associated to a mitral valve insufficiency, detected with radiographic and echocardiographic examination. Two ferrets (2/6) presented heart murmur without pulmonary signs, and mitral valve insufficiency was detected with echocardiographic examination. Abnormal hyperechoic structures in the right atrium, in right ventricle or in cranial vena cava were detected. Moreover, significant correlation was found between *D. immitis* seropositivity and the presence of clinical signs associated to heart disease ($p=0.02$) and respiratory disease ($p=0.03$), and the presence of clinical signs compatible with heart-respiratory disease ($p=0.008$).

To the authors´ knowledge, the present study is the first report of *D. immitis* antibody detection in domestic ferrets in Spain. The information on the distribution of *D. immitis* and epidemiology of heart worm 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 in dogs in the Valencian region is 6.95%, based on results of immunochromatographic tests of *D. immitis* antigens (Montoya-Alonso et al., 2020).

The clinical features of feline heart disease make difficult to diagnose the disease in cats, and also in domestic 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 (Villanueva-Saz et

187 al., 2021; Montoya-Alonso et al., 2014). The present study focused on the *D. immitis*
188 infection in domestic ferrets, and detected similarly high, 14.51% seroprevalence in
189 household domestic ferrets from the Province of Valencia, on the east coast of the Iberian
190 Peninsula.

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

205 No significant correlation was found between positivity for anti-*D. immitis* antibodies and
206 the gender of ferrets. Although dirofilariosis is transmitted by infected mosquitos, 17.20%
207 of the seropositive (16/93) ferrets live indoors. The seropositivity was notably higher in
208 indoor ferrets (17.20%) than in the outdoor ones (9.09%). The examined ferrets lived both
209 indoor and outdoor were seropositive in 12.68% (9/71). One possible explanation of the
210 high seropositivity in indoor ferrets compared to the outdoor lifestyle is the fact that
211 human activity is able to create environments to facilitate the proliferation of vectors,

some of them with heartworm larvae. Moreover, it is described that cats with indoor lifestyle does not protect from infection (Montoya-Alonso et al., 2014).

The results of this study show the need for the implementation of preventive measures including chemoprophylaxis against mosquito throughout the transmission period to avoid the interaction with the ferret 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 these 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 of 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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329 Table 1**.** Serological results of *D. immitis* infection in ferrets examined, and its
330 correlation to various factors.

Examined factors		*Dirofilaria*-specific antibodies by ELISA		
		No. of positive/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 yrs)	2/41	4.88	No (p=0.052)
	Adult (2–6 yrs)	22/127	17.32	
	Senior (>6 yrs)	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 compatible with heart-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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