Veterinary Parasitology: Regional Studies and Reports 23 (2021) 100541

Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr

Original Article

Prevalence of microfilariae, antigen and antibodies of feline dirofilariosis infection (*Dirofilaria immitis*) in the Zaragoza metropolitan area, Spain

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ARTICLE INFO

Keywords:
Dirofilaria immitis
Feline
Heartworm
Seroprevalence studies
Serology
Vector-borne diseases

ABSTRACT

Feline heartworm disease is a vector-borne parasitical disease caused by *Dirofilaria immitis*. Heartworm infection in dogs is prevalent in the Mediterranean countries. Information about the geographical distribution and epidemiological features of *D. immitis* infection in cats is scarce, particularly in urban stray cats that live within endemic regions for canine heartworm disease. The aim of the current study was to determine the seroprevalence of antigen and antibodies to *D. immitis* in feral cats in Zaragoza city, an endemic region of Spain. For this purpose, blood samples were examined for microfilariae using a direct blood smear technique and the modified Knott test. Two serological techniques for anti-*D. immitis* antibody detection (Solo Step® FH and in-house ELISA) and three different commercial antigen tests (DiroChek®, MegaELISA® DIRO Antigen and *FAST*est® HW) were performed. Blood samples from 250 stray cats were tested: 61 cats (24.40%) tested positive by the in-house ELISA, and 9 cats gave positive (3.6%) results with Solo Step® FH. The global seroprevalence of *D. immitis* in the feline population of the studied area of Zaragoza was 25.20% (63/250) including Solo Step® FH result and in-house ELISA. The blood exam for all samples was negative when evaluating for microfilariae and not a single cat was positive for antigen testing. This study demonstrates the presence of *D. immitis* infection in Zaragoza city. Veterinarians working in endemic areas should be aware of this infection in cats at risk and their susceptibility.

1. Introduction

Dirofilariosis is a globally distributed vector-borne disease globally distributed caused by *Dirofilaria immitis* and transmitted by culicid mosquitoes under natural conditions during feeding (McCall et al., 2008). The importance of the spread of *D. immitis* and other feline vector-borne pathogens is related to climate change with a direct effect on the vector's ecology (Morchón et al., 2012). Furthermore, animal mobility between geographical areas, especially dogs and cats from endemic areas or pets that travel from endemic to non-endemic areas, potentially spread these vector-borne infections (Simón et al., 2012).

Various species of culicid mosquitoes have been involved in the transmission of heartworm disease in European Mediterranean countries (Cancrini et al., 2006). Dogs are considered the main reservoir host for *D. immitis* but other domestic animals can act as potential hosts but not as a reservoir, such as cats (Simón et al., 2012) and ferrets (McCall, 1998). In the same way, wild carnivores (Simón et al., 2012; Penezić et al., 2014) and mustelids (Matsuda et al., 2003; Penezić et al., 2018) have been considered as silent reservoirs of *D. immitis* in different geographical areas of the world.

Cats usually have a low parasite burden, but mono-sexual infections are also quite frequent (Genchi et al., 2008; Dillon et al., 2017a). Differences between dogs and cats have been described considering pathogenesis and clinical presentation. This variability between species is thought to be the result of immune response from the host against the parasite and bacterial endosymbiont *Wolbachia*, which may be influenced by individual factors of the host (Morchón et al., 2004; Kramer et al., 2005; García-Guasch et al., 2013). Compared to dogs, the disease in cats has mainly a pulmonary presentation, whilst the disease progresses in dogs, cardiac involvement occurs (Browne et al., 2005). The most common clinical signs of feline dirofilariosis include respiratory and digestive signs such as dyspnea, tachypnea, coughing, vomiting and

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https://doi.org/10.1016/j.vprsr.2021.100541
Received 3 December 2020; Received in revised form 12 January 2021; Accepted 22 January 2021
Available online 28 January 2021

diarrhea. Other non-specific clinical signs detected during physical examination are generalized weakness, weight loss and anorexia (Pennisi et al., 2020). By contrast, the arrival and death of immature adult *D. immitis* in the pulmonary systems induced a heartworm-associated respiratory disease detecting rapid heart rate, blindness, collapse, convulsions and finally sudden death (Dillon et al., 2014; Dillon et al., 2017b).

The diagnosis of feline dirofilariosis is relatively more complex in comparison to the canine heartworm identification because infected cats have low worm burden with the absence of microfilaremia (Venco et al., 2015; European Society of Dirofilariosis and Angiostrongylosis, 2017; Pennisi et al., 2020). This is reflected by the employ of serological techniques to carry out the fairly complex diagnosis in cats.

Feral colonies are often found in European cities and contact between cats and humans could aid in the transmission of zoonotic agents causing health risks. Notably, feral cats are animals that could be considered as potential sentinels for vector and pathogen environmental pressure. Therefore, free-range lifestyle is associated with a higher exposure to vector-borne pathogens, making an impact in the epidemiology of the disease.

The detection of vector-borne pathogens is becoming more common in dogs and cats. Some zoonotic diseases such as *Leishmania infantum* have been frequently detected in dogs and cats (Bourdeau et al., 2014; Pennisi et al., 2005), further surveillances including *D. immitis* have been less extensively investigated in domestic and feral cats. No epidemiological studies investigating *D. immitis* in cats had been previously conducted in feline colonies from Spain. The goal of the study was to determine the current feline heartworm seroprevalence in feral cats from the Zaragoza metropolitan area and the potential inherent risk factors associated with the heartworm infection.

2. Material and methods

2.1. Study areas, cats and sampling

The study was carried out in the metropolitan area of Zaragoza city (41° 39′ 24.6276" N, 0° 52′ 45.912" W). Located in the Ebro Valley region of northeast Spain, a highly endemic heartworm disease area. The region extends from 177 to 741 m above sea level. Some rivers basins including The Huerva River and The Gállego River cross the territory carrying the water flow to the Ebro River. It is the second longest river in the Iberian Peninsula, after the Tajo River and the longest river running entirely within Spain. With a semiarid climate with of dry, hot summers and cold winters, precipitation is not abundant being more frequent in Spring and the dry season continues beyond the end of summer.

The study population comprised 250 stray cats captured in urban areas of Zaragoza within a trap, neuter, and release sterilization program run locally to control stray populations, from November 2017 to November 2019. Cats included in this study were at least 1-year-old, had received no heartworm prevention or long-acting topical insectide, and had lived in the same feline colony city.

This survey was included under Project Licence PI62/17 approved by the Ethic Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed according with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Cats were anesthetized with a combination of medetomidine and ketamine. Information about breed, age, gender and colony of origin was recorded. A complete physical examination was carried out before sampling but fecal examinations were not performed. For each feline patient, three mL of blood was collected aseptically by jugular vein puncture and divided equally into a sterile blood collection tube to obtain the serum and another tube containing EDTA. Separated serum were stored at −20 °C until use.

2.2. Diagnostic tests

2.2.1. Detection of the presence of microfilariae in fresh blood samples

A direct blood smear technique and the modified Knott test were performed to detect the presence of microfilariae in fresh blood samples (Marcos et al., 2016).

2.2.2. Detection of circulating D. immitis antigens by ELISA and immunochromatographic rapid test

Circulating *D. immitis* antigens were detected by using two different commercial ELISA kits (DiroChek® Heartworm Antigen Test Kit, Zoetis, Florham Park, USA; MegaELISA® DIRO Antigen, Megacor Diagnostik, Hörbranz, Austria), and an immunochromatographic rapid test (FASTest® HW, Megacor Diagnostik, Hörbranz, Austria), following the manufacturer's instructions. In the case of ELISA technique, colour development indicates the presence of heartworm antigen in the sample. By contrast, samples with a clear test and control line are classified as positive and samples that show a control line are classified as negative for the immunochromatographic test. Each test used in this study was performed by a different researcher without knowledge of the results of the rest of the tests.

2.2.3. Detection of D. immitis antibodies by immunochromatographic rapid test and in-house ELISA

Anti-*D. immitis* antibodies were detected using two different tests including a commercial immunochromatographic rapid test (Solo Step® FH, Heska, Loveland, Colorado, USA) and an in-house ELISA being the well coated with recombinant antigen of *D. immitis* (Urano Vet®, Barcelona, Spain). In brief, the plates were coated with 0.5 μg of *D. immitis* pepsin inhibitor Dit33 (DIT33) recombinant protein. Serum samples were prepared at 1/100. Anti-feline IgG antibody, horseradish peroxidase-labelled (Bethyl laboratories, Montgomery, USA), was applied at 1/20000 dilution. The optical densities were measured in a microplate reader (ELISA Reader Labsystems Multiskan, Midland, Canada) at 450 nm. Taking the negative control OD450 (Optical Density 450) value two cut-off points were calculated: the negative cut-off (OD450 negative control +0.25) and the positive cut-off (OD450 negative control +0.30). The samples with an OD higher than the positive cut-off were classified as positive. The samples with an OD lower than the negative cut-off were classified as negative. The samples with an OD between the two cut-offs were classified as doubtful. The positive cut-off for all experiments considering mean + standard deviation was 0.35 ± 0.003 whilst, the negative cut-off (mean + standard deviation) was 0.30 ± 0.003. Sera were classified as being high positive, when having an OD equal or higher than 0.800 (≥0.800), medium positive were classified as OD equal or higher than 0.400 (>0.400) and less than 0.800 (<0.800). Finally, low positive were sera from those cats with OD lower thank 0.400 (<0.400) and higher than the positive cut-off. The in-house ELISA was validated using 12 sera infected by *D. immitis* from an experimental study. The sera were provided by TRS Labs (GA, USA), These samples contain a variable number of female and/or male worms. Moreover, they were evaluated for 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 a positive result for all tests. Each test used in our study was performed by a different researcher without knowledge of the results of the rest of the tests.

2.3. Statistical analysis

Descriptive statistics were used to demonstrate heartworm seroprevalence. Associations between percentage of positivity rates and the variables recorded (gender and colony of origin) were analyzed. The significance of differences was assessed using the chi-square or Fisher's exact test. A $p \leq 0.05$ was considered significant. The SPSS program (SPSS Inc., Chicago, USA) was used.

3. Results

3.1. Global results

In this study, there were 110 male and 140 female cats, shorthair type and more than one year old. All cats were classified as apparently healthy animals without any evident clinical signs making them fit for the surgical sterilization procedure. Each cat was part of a feline colony established in Zaragoza city and these animals had never received any treatment for heartworm disease. The cats were analyzed according to the proximity of the feline colony to river basins of the three rivers in Zaragoza: Ebro, Huerva and Gállego. Regarding the type of environment, all cats came from colonies close to river basins. No significant association was found between positivity for anti-*D. immitis* antibodies and gender nor colony of origin ($p > 0.05$).

3.2. Positive results for both microfilaria and antigens

No positive result was obtained by antigens tests (0/250), the modified Knott test (0/250) and the direct blood smear technique (0/250).

3.3. Positive results to each antibody test

The global seroprevalence of *D. immitis* in the feline population of the studied area of Zaragoza was 25.20% (63/250) including Solo Step® FH result with 3.6% (9/250) and in-house ELISA with 24.40% (61/250).

Two seropositive samples by Solo Step® FH were seronegative by the in-house ELISA. Regarding sex, 22.72% of males (25/110) and 25.71% of females (36/140) were seropositive by the in-house ELISA, whilst, the Solo Step® FH detected 1.82% of males (2/110) and 5% of females (7/140).

Considering the in-house ELISA results, seropositive samples in the present study had different antibody levels, being 18 samples classified as low positive, 35 samples as medium positive and finally 8 samples as high positive. Whilst, seven samples were classified as doubtful result.

3.4. Positive results to both antibody tests

Of the seropositive samples detected by Solo Step® FH, 7 samples showed concordant results between this rapid test and the in-house ELISA. These samples were classified as high positive ($n = 2$), medium positive ($n = 4$) and low positive ($n = 1$) by the in-house ELISA.

4. Discussion

The present study represents the first serosurvey of *D. immitis* carried out in a feral cat population performed in a Spanish city which classifies as an endemic area for this infection. In this context, the antibody seropositivity does not show an actual prevalence such as presence of immature and/or mature worms, but it is an indirect way to determine the risk of heartworm infection.

This infection has been documented in cats from the Iberian Peninsula including Portugal and Spain, although the information is limited. In Portugal, two studies have demonstrated the presence of the infection in cats. In a study conducted in central and northern Portugal, blood samples from 434 client-owned cats were evaluated to detect the presence of anti-*D. immitis* and anti-*Wolbachia* surface protein (WSP) antibodies, obtaining an overall feline seroprevalence of 15% (Vieira et al., 2015). A second study has been published and the seroprevalence of *D. immitis* antigens was 4.8% after evaluating 271 mixed cats including domestic and stray cats from southern Portugal (Maia et al., 2015). In Spain, this infection has been detected in cats from continental Spain and the Canary Islands. The most recent study conducted was in the Madrid Province, 531 client-owned cats living in the metropolitan area of Madrid and near areas were tested for *D. immitis* antigens, anti-*D. immitis* antibodies and anti-WSP antibody detection tests (Montoya-Alonso et al., 2017). As a result, 0.2% were positive to the circulating antigens and a total of 7.3% of the cat's antibodies against *D. immitis* and *Wolbachia* were detected. A similar result (0.26%) considering the presence of circulating *D. immitis* antigens was obtained after examining 758 client-owned cats from the Barcelona metropolitan area (Montoya-Alonso et al., 2014). However, *D. immitis* and WSP antibodies were higher (11.47%) in comparison to Madrid Province (Montoya-Alonso et al., 2017). From non-continental Spanish region, the Canary Islands, an hyperendemic area of dirofilariosis from 707 client-owned cats, showed a seroprevalence of feline dirofilariosis of 18.1% with a positive result to both anti-*D. immitis* and WSP antibodies (Montoya-Alonso et al., 2016).

In our study, the type of the serological technique performed may influence the seroprevalence data obtained with higher seroprevalence when using a quantitative serological technique in comparison to a qualitative rapid test that identified exposed animals. The difference between Solo Step® FH result with 3.6% (9/250) and in-house ELISA with 24.40% (61/250) could probably be caused by false positivity results due to cross reactivity of the more sensitive test as a possible bias. In our study, cross reactivity with other gastrointestinal parasites was not assessed because no fecal examinations were performed. False positivity results may also be the consequence of non-specific antibody reactions, the presence of ectopic infections and the persistent concentrations after the death of worms including adult forms and immature larvae. Other circumstances that could have an influence in diagnostic results between tests are the technology, test threshold and type of antigen. In the case of the type of antigen, Solo Step® FH is composed by rHWAg1 recombinant antigen, sequenced from antigens that can be found in microfilaria, L3 and L4 larvae and adult worms. By contrast, the in-house ELISA uses a recombinant antigen named DIT33, sequenced from antigens of microfilaria, L4 larvae, and adult worms. Moreover, other determinants including differences in the populations studied and the geographical area analyzed (areas close to rivers is a risk factor) have a direct influence to determine the seroprevalence.

In dogs, different diagnostic techniques have been available including several commercial tests that detect the presence of circulating antigens and the modified Knott test. By contrast, the presence of circulating antigens is a recommended diagnostic technique to use in cats, despite low sensitivity. Also, cats are generally more resistant to infection than dogs. In this sense, the detection of anti-*D. immitis* antibodies could be interesting to demonstrate exposure to the parasite rather than an active infection. An antibody positive result confirms that the cat is at risk, and that detected antibodies may indicate a mature infection, ongoing exposure to heartworm larvae, or previous exposure (Atkins et al., 2000).

The overall seroprevalence of *D. immitis* found in this study was apparently higher than the ones obtained in domestic cats from Barcelona and Madrid, which might be related with the fact that all animals included in this study were stray cats and thus lived outdoors lifestyle in areas close to rivers basins in contrast to client-owned cats from the other Spanish studies. This situation is very similar in dogs and the seroprevalence of canine heartworm disease is different depending on the lifestyle being the dog with a complete outdoor lifestyle the highest seroprevalence rate in comparison with dogs with >50% outdoor lifestyle, following by dogs with dogs with >50% indoor lifestyle and finally dogs with 100% indoor lifestyle (Lu et al., 2017).

In hyperendemic areas for heartworm infection, feline prevalence is within the limits of 10% of the prevalence of dogs (Venco et al., 2011). In our study, the feline seroprevalence was higher (25.20%) in comparison with the canine prevalence detected previously (13.5%) (Castillo et al., 1989). The reason for this discordance could be associated to the temporal separation between the results of dogs and cats. Therefore, further epidemiological studies including dogs from Zaragoza city should be considered. There are not any recent studies with this information except for large epidemiological study performed in Spain but the results

obtained are not comparable (Montoya-Alonso et al., 2020). These results are in agreement with other studies published in central and northern Portugal and Gran Canarias. Based on the results obtained in our study of seroprevalence, we confirm that heartworm exposure in stray cats is widely present in Zaragoza city and we believe that all cats are at risk for heartworm exposure. To corroborate our results, other studies including feral cats should be performed in different Spanish or European cities.

Stray cats represent a sentinel population for a variety of infections, because they receive no prophylaxis and are continually exposed to vector-borne pathogens. The importance of this study is that the results gives an idea of the infection risk, different from other studies that include cats receiving veterinary care where may have occurred an underestimated feline heartworm infection risk may have occurred. Despite the high number of exposed cats, some of these cats with high and moderate anti-*D. immitis* antibodies levels and no evident clinical signs prior the surgical procedure, could explain the resistance to the infection and the asymptomatic course of feline dirofilariosis.

One of the most common problem associated to detection of the presence of anti-*D. immitis* antibodies is the lack of different serological techniques apart from the only commercial rapid test available. Most of the serological studies performed in Spain are based on ELISA technique using two types of antigens including a pool of synthetic peptides derived from two molecules present in *D. immitis* adults worms called Di22 and Di33 and, a recombinant protein from the surface of the endosymbiont *Wolbachia*. In this sense, we are developed an in-house ELISA test based on pepsin inhibitor DIT33 recombinant protein.

One limitation of this study was the lack of clinical information of exposed cats due to the difficulty of doing a complete clinicopathological examination and other additional diagnostic tests, such as echocardiography and thoracic X-ray, for a better clinical characterization of these animals. Another limitation that should be considered is that no antigen positive result was obtained, and the heat treatment technique was not used, which would likely have had an impact on the final results (Szatmári et al., 2020).

5. Conclusion

Our study demonstrated the presence of anti-*D. immitis* antibodies in apparently healthy stray cats in Zaragoza using two different techniques including a rapid immunochromatographic test and in-house ELISA. This is the first seroepidemiological survey describing *D. immitis* exposure in stray cats in Spain. Veterinarians working in endemic areas should be aware of this infection in cats at risk and their susceptibility. Further research is needed to better understand the epidemiological role of stray cats in urban areas throughout the study of microfilaraemic hosts, usually it is absent or sporadic in cats.

Ethical statement

This survey was included under Project Licence PI62/17 approved by the Ethic Committee for Animal Experiments for the University of Zaragoza. The care and use of animals were performed according with the Spanish Policy for Animal Protection RD 53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgements

The authors thank Maria Ángeles Lostao and Irene Fernández (Biotechnology student) for technical support on serological testing. We would like to acknowledge all veterinarians participating in this study.

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