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Serum acute phase proteins in *Dirofilaria immitis* and *Wolbachia* seropositive cats

Ana C Silvestre-Ferreira[1,2], Lisete Vieira[1], Hugo Vilhena[2,3,4], Jose J Cerón[5], Asta Tvarijonaviciute[5,6], José A Montoya-Alonso[7], Elena Carretón[7] and Josep Pastor[6]

Abstract

Objectives The aim of this study was to characterise the response of acute phase proteins (APPs) in cats seropositive for *Dirofilaria immitis* and to its endosymbiont bacterium *Wolbachia*.

Methods The APPs serum amyloid A (SAA), haptoglobin (Hp) and ceruloplasmin (Cp) were measured in 25 seropositive cats and in 16 healthy seronegative cats.

Results SAA and Cp concentrations were significantly higher in animals with *D immitis* seropositivity that exhibited clinical signs related to the disease, and Hp was elevated in all *D immitis*-seropositive animals. There was no significant correlation between APPs and *D immitis* or *Wolbachia* species antibody titres.

Conclusions and relevance An association between feline seropositivity to *D immitis* and APP response was demonstrated. Increases in serum SAA and Cp concentrations were related to *D immitis*-associated clinical signs, whereas Hp increased in all seropositive animals.

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Introduction

Feline dirofilariosis is caused by the intravascular parasite *Dirofilaria immitis*, which is transmitted by culicid mosquitoes.[1] Infection with *D immitis* is preventable; however, once an animal is infected, it can develop heartworm disease and potentially life-threatening complications. Contrarily to dogs, in cats right-sided heart failure and caval syndrome have been rarely documented.[2] In cats, the majority of the immature worms that reach the caudal pulmonary arteries die. This produces a strong vascular and parenchymal inflammatory response approximately 3 months after infection, inducing clinical signs such as coughing, dyspnoea or intermittent vomiting. In chronic feline heartworm infection, the adult heartworms suppress the function of pulmonary intravascular macrophages, reducing the severity of the clinical disease.[2–4] Clinical presentation varies from asymptomatic infections to chronic respiratory signs, sometimes accompanied by chronic vomiting and even acute death with no premonitory signs.[2] Owing to the unspecific clinical presentation, the low worm burdens and the weak levels of circulating antigens, diagnosis can be challenging and relies on a combination of tests, such as serological and parasitological tests, thoracic radiography and echocardiography.[2,4]

Although cats appear to be more resistant than dogs to adult *D immitis* infection, feline dirofilariosis has been

[1]Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
[2]Animal and Veterinary Research Centre (CECAV), University of Trás-os-Montes and Alto Douro, Vila Real, Portugal
[3]Department of Veterinary Medicine, Vasco da Gama University School Coimbra, Coimbra, Portugal
[4]Baixo Vouga Veterinary Hospital, Segadães-Águeda, Portugal
[5]Interdisciplinary Laboratory of Clinical Analysis (Interlab-UMU), Veterinary School, Campus of Excellence Mare Nostrum, University of Murcia, Espinardo, Murcia, Spain
[6]Department of Animal Medicine and Surgery, Autonomous University of Barcelona, Barcelona, Spain
[7]Research Institute of Biomedical and Health Sciences (IUIBS), Faculty of Veterinary Medicine, University of Las Palmas de Gran Canaria, Arucas, Las Palmas, Spain

Corresponding author:
Ana C Silvestre-Ferreira DVM, PhD, Department of Veterinary Sciences, University of Trás-os-Montes and Alto Douro, 5000-801 Vila Real, Portugal
Email: aferreir@utad.pt

diagnosed worldwide. Studies performed in canine endemic areas show high feline seroprevalences, indicative of infection risk in cats.[5,6]

Many filarial species, including *D immitis*, harbour intracellular *Wolbachia* species, an endosymbiont bacterium.[1,7] The presence of circulating *Wolbachia* species antibodies and *Wolbachia* species antigens in tissues of infected animals have been associated with inflammation.[8,9]

Serum acute phase proteins (APPs) are considered highly sensitive biomarkers of inflammation that can be of use in the diagnosis, management and prognosis of a variety of clinical conditions.[10,11] In the cat, serum amyloid A (SAA) is considered a major APP, increasing a few hours after the inflammatory stimulus and remaining elevated for as long as the inflammation persists; haptoglobin (Hp) and ceruloplasmin (Cp) are considered moderate APPs as their increment is moderate and slower, returning to normal values with a more gradual decline.[10]

It could be postulated that combining APP and *D immitis* seropositivity could increase the index of suspicion of *D immitis* as the cause of clinical signs. This survey aims to study APP response in *D immitis*-seropositive cats. For this purpose serum SAA, Hp and Cp concentrations were studied in seropositive cats which were divided into three groups: asymptomatic, symptomatic with clinical signs compatible with *D immitis* and symptomatic with signs not compatible with *D immitis.* The influence of *D immitis* titres and *Wolbachia* endosymbiont antibody titres on APP concentrations was also assessed.

Materials and methods

The SAA, Hp and Cp levels were determined in the sera of 41 cats, obtained from previous research. The study was approved by the ethical committee of the Veterinary Medicine Service of University of Trás-os-Montes and Alto Douro and was carried out in accordance with the current European legislation on animal protection. All owners were informed and gave informed consent to permit the utilisation of the blood samples of their animals. *Dirofilaria immitis*-seropositive cats were identified using serological techniques for anti-*D immitis* and anti-*Wolbachia* antibody detection,[6] and all cats were tested with real-time polymerase chain reaction (PCR) for *Hepatozoon felis, Hepatozoon canis, Leishmania infantum* and agents of the genera *Babesia, Ehrlichia, Anaplasma, Rickettsia* and *Mycoplasma*.[12] Of them, 25 cats were seropositive to anti-*D immitis* and anti-*Wolbachia* antibodies, and 16 were seronegative cats that were considered healthy on physical examination (control animals). All the included animals were negative to the PCR-tested agents.

Seropositive animals were classified as asymptomatic (group 1; n = 9); symptomatic with clinical signs related to *D immitis* such as anorexia, weight loss, cough, dyspnoea and vomiting (group 2; n = 12); and symptomatic with clinical signs triggered by other diseases (renal disease, otitis, pyothorax, limb abscess) not related to *D immitis* (group 3; n = 4). This classification was made by an experienced internist who was blinded to the APP data before assigning cats to the groups.

The SAA concentrations were determined by a human turbidimetric immunoassay (LZ-SAA; Eiken Chemical) performed on an automated analyser (Olympus AU2700; Olympus Diagnostica).[13] Serum Hp concentrations were determined by the haemoglobin binding method with the use of a commercial kit (Tridelta Development).[13] Cp concentrations were determined by a method previously described.[14] The Hp and Cp analyses were performed in a biochemical autoanalyser (Cobas Mira Plus multi-parametric autoanalyser; ABX Diagnostic). All the methods showed inter- and intra-assay imprecision lower than 15%, and the dilution of the samples resulted in linear regression equations with a correlation coefficient close to 1.

The normal distribution of the studied parameters was accessed by a Kolmogorov–Smirnov test. Because the parameters did not follow a normal distribution, a Kruskal–Wallis test was used to assess differences in concentrations between groups. Mann–Whitney post-tests with type I error correction were used whenever statistically significant differences were observed. A Spearman correlation test was used to study a possible link between anti-*D immitis* and anti-*Wolbachia* antibodies, and APP. Statistical significance was set at $P < 0.05$.

Results

The values of the measured APPs in all groups are shown in Table 1. Serum SAA and Cp concentrations were significantly higher in cats of group 2 (*D immitis*-seropositive animals that showed compatible clinical signs) when compared with group 1 or group 3 animals ($P < 0.05$). Group 3 animals (symptomatic animals with diseases not related to *D immitis*) showed similar serum concentrations of SAA and Cp to those of the control cats. Hp was statistically significantly higher in seropositive cats (groups 1, 2 and 3) when compared with control animals ($P < 0.05$).

The anti-*D immitis* and anti-*Wolbachia* antibodies titres from group 1 (asymptomatic cats) and group 2 (cats showing clinical signs) did not show statistically significant differences in seropositivity. No statistically significant correlation was observed between antibodies anti-*D immitis* or anti-*Wolbachia* titres and APP concentrations.

Discussion

The aim of the present work was to evaluate the APP profile in *D immitis*-seropositive cats through the use of a major (SAA) and two moderate APPs (Hp, Cp).

Table 1 Anti-*Dirofilaria immitis* and anti-*Wolbachia* antibodies titres, and concentrations of serum amyloid A (SAA), haptoglobin (Hp) and ceruloplasmin (Cp) in healthy (control) and infected cats

	Control	Without clinical signs (group 1)	Compatible clinical signs (group 2)	Not related clinical signs (group 3)	Groups comparison (Kruskal–Wallis *P* value)
n	16	9	12	4	
Anti-*D immitis* antibody titres	ND	0.757 (0.556–0.891)	0.811 (0.708–0.969)	0.797 (0.701–0.903)	0.347
Wolbachia titres	ND	0.697 (0.560–0.935)	0.777 (0.600–0.962)	0.6635 (0.623–0.724)	0.217
SAA (mg/l)	0.3 (0.0–0.6)[a]	0.4 (0.0–8.2)[a]	30.2 (0.0–121.0)[b]	1.05 (0.30–58.1)[a]	0.001
Hp (g/l)	1.51 (0.35–2.30)[a]	2.94 (1.17–10.00)[b]	3.31 (1.97–15.40)[b]	3.88 (1.33–10.10)[b]	<0.001
Cp ($_{\Delta abs}$ /min $\times 10^{-3}$)	4.5 (0.3–54.0)$_a$	5.5 (0.4–7.1)[a]	9.4 (5.6–55.0)[b]	5.2 (3.0–5.5)[a]	<0.001

Data are mean (interquartile range). Cp results are reported as the change in absorbance per minute at 550 nm. Controls were seronegative to *D immitis*. All samples from the groups 1–3 were seropositive to *D immitis*
Different letters between parameters indicate statistical significance ($P < 0.01$)
ND = not detected

The results obtained indicated that serum SAA was elevated only in cats showing clinical signs compatible with *D immitis* infection (group 2). Similarly, previous reports found that SAA concentration increased significantly in the acute phase but returned rapidly to baseline levels after a cat's experimental infection with either *Mycoplasma haemofelis* or '*Candidatus* Mycoplasma haemominutum'.[13] Furthermore, only C-reactive protein (CRP), a canine major APP,[10] showed significant differences between asymptomatic and symptomatic dogs infected with *D immitis*;[15] and in human medicine major APPs such as CRP, SAA and Hp were found to be significantly elevated in patients with active *Wuchereria bancrofti* and *Brugia malayi* infection (the lymphatic-dwelling filariae disease).[16]

Pulmonary inflammation caused by *D immitis* may account for the increase in serum SAA in group 2 animals. Previous reports described respiratory signs in cats as attributable to the death of both juvenile and adult worms within the pulmonary arteries.[3] Although, there seems to be evidence that *Wolbachia* species produces a greater acute inflammatory response, worsening the bronco-reactivity associated with *D immitis* seropositivity in cats,[17] in our study *Wolbachia* titres were similar between clinically affected and non-affected *D immitis*-positive animals.

All groups of cats seropositive for *D immitis* showed elevation in serum Hp concentration in comparison with seronegative healthy animals. However, in dogs naturally infected with *D immitis*, Hp was not increased in positive animals and even decreased in those presenting microfilaraemia. We hypothesised a connection between this decrease and the possible presence of haemolytic anaemia;[15] however, no signs of haemolytic anaemia were found in the cats of our study. The reason for the increased serum concentrations of Hp in the present study is unknown. Although Hp levels can increase during inflammation unrelated to heartworm infection and therefore is not specific to this disease, it could be postulated that if increased Hp concentrations are detected in cats living in endemic areas, *D immitis* infection should be included in the list of differential diagnoses.

Increased serum Cp levels were only observed in seropositive cats with clinical signs related to *D immitis* infection. Although Cp is also related to oxidative stress,[10] current knowledge on the level variations in feline inflammatory conditions is limited. Substantial further investigation is needed in order to determine its potential utility in cats.

A major limitation of this study is that antibody titres only confirm *D immitis* exposure – seropositive cats are not necessarily infected. In addition, studies involving a larger number of animals to determine the sensitivity and specificity and optimal cut-off points of the different APPs for the detection of animals with clinical signs would be desirable. In the present study no significant differences were observed between *D immitis* and *Wolbachia* antibody titres and the presence/absence of clinical signs. Based on the results, it could be postulated that disease activity of *D immitis* be assessed by using the APP profile combined with evaluation of antibody levels when clinical signs and complementary diagnostic examinations suggest heartworm infection. These data are in accordance with studies in *Leishmania*-infected dogs, which suggest that CRP concentration reflects disease activity better than antibody concentration.[18] Also, the determination of *Wolbachia* species titres is not likely

to be useful for disease activity assessment, as, in this study, there was no evidence that exposure to these bacteria was associated with worse clinical signs.

Conclusions

To our knowledge, this is the first study to evaluate comprehensively the APP response of cats seropositive for anti-*D immitis* and anti-*Wolbachia* antibodies. Although precautions should be taken, owing to the relatively low number of animals included, the results show an association between feline seropositivity for *D immitis* and an APP response. Increases in SAA and Cp are related to *D immitis*-associated clinical signs, whereas Hp increases in seropositive animals regardless of the presence or absence of clinical signs.

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