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Research Article

Use of biomarkers as prognostic indicators in dogs with natural heartworm

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Abstract. Heartworm disease is a parasitic illness caused by the *Dirofilaria immitis* nematode. In its developed form, remains in the pulmonary artery and right side of the heart, resulting in pulmonary thromboembolism, myocarditis, and inflammation. A retrospective study was made in which was evaluated the usefulness of the Dimer-D, troponin I and C-reactive protein in sick dogs naturally infected with heartworm. There were evaluated the concentrations of D-Dimer, troponin I and C-reactive protein in 23 dogs, analyzed hematological variables, the presence or absence of microfilariae, the pulmonary hypertension and clinical signs. The respiratory problems were the most frequent clinical signs including dyspnea (74%), cough (30%), pulmonary hypertension (57%), and other signs of inflammation or pulmonary thromboembolism. Hematological changes were not found. Elevations of the Dimer-D were found in 73.9% of cases, where the patients with microfilariae (69.6%) showed higher values compared to amicrofilaremics (30.4%); males had a higher average (3,857.83 ng ml-1) compared to females (1,714.0 ng ml-1). Troponin I and C-reactive protein had elevations in 21.7 - 39.1% of cases without significant changes compared to sex or microfilariae. The measurement of Dimer-D, troponin I and C-reactive protein complements for the diagnosis, prognosis and therapeutic control in patients with *D. immitis* indicated inflammation, pulmonary thromboembolism and/or myocarditis.

Key Words: pulmonary hypertension, acute phase proteins, troponins.

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Introduction

Pulmonary thromboembolism (PT) is the formation of clots in the pulmonary arterial circulation (Lynelle et al 1999). Dirofilariosis is also known as heartworm disease and is caused by a parasite of the genus *Dirofilaria immitis*, which in adult phase is located in the pulmonary artery or in its branches (McCall et al 2008). The adult parasite is in contact with the vascular endothelium triggering a proliferative endoarteritis, ending with the production of thrombi; the death of the parasites or fragments of them travel to the smaller branches of the lungs triggering PT, these effects leading to the development of pulmonary hypertension (PH) (Simón et al 2012). The presence of PT and PH causes damage to the myocardium due to volume and pressure overload (Goggs et al 2017).

The use of biomarkers has grown in veterinary medicine, particularly in patients with *D. immitis* (Carretón et al 2011). Troponin I (nTcI), for example is one of the myofibrillar proteins that regulates the interaction of calcium with myosin and actin. Its blood elevation correlates with myocardial injury or myocyte necrosis. It has high specificity and sensitivity as a cardiac biomarker (Darcy et al 2005) (Becattini et al 2007). D-dimer is generated during thrombus formation when the factor XIIIa cross-links the terminal D domains of fibrin (Tracy, 2002). The increase in its concentrations is present in dogs with PT (Epstein et al 2013), but it should be noted that this increase is not useful in order to verify the formation of clots, due to their low specificity (Nelson et al., 2003) (Janata et al 2003). The C-reactive protein (CRP) is an acute phase protein that helps restore homeostasis and limit bacterial growth independently of antibodies in response to inflammation, infection or trauma (Murata et al 2004) (Kumar et al 2015). These proteins are used for diagnosis, prognosis and therapeutic monitoring; it is highly sensitive, indicating inflammatory processes, but is not very specific (Eckersall et al 2010). It is the most used acute phase protein in dogs and is reported that it alters in the presence of *D. immitis* (Méndez et al 2014). The general objective of the present report was to evaluate the use of cardiopulmonary and inflammatory biomarkers (cTnI, D-dimer and CRP) in dogs infected with *D. immitis* as prognosis in the presentation of PT, myocarditis and inflammation, and to perform an adequate therapeutic plan.

Materials and methods

The medical records of the Mastervet Veterinary Clinic in the city of Barranquilla (Colombia) were used to perform a retrospective study on patients with heartworm disease from January 2016 to January 2017. During this period, 54 individuals were positive. However, only 23 clinical reports were analyzed, since they included cardiopulmonary and inflammatory biomarkers as

a complement for the diagnosis of PT, myocarditis and inflammation. The parameters that were evaluated included: clinical history, laboratory tests, existence of microfilariae, presence of pulmonary hypertension and survival.

The diagnosis was made through ELISA commercial test that detects circulating antigens (antigen rapid heartworm Ag test kit 2.0, Bionote Laboratories, Republic of Korea) according to the manufacturer's specifications. The presence or absence of microfilariae was evaluated in positive dogs using Knott's modified test. For the diagnosis of PH, the patients underwent echocardiogram with spectral and color Doppler (Mindray Z6Vet) placing them in right lateral recumbency with the transducer placed in the third intercostal space; the reports were carried out by the same person. The age range of the studied individuals was from 2 to 10 years old (mean of 5.26), including 5 females and 18 males.

Blood samples were taken from the jugular or cephalic vein in each patient. For the measurement of cTnI and D-dimer were used red and blue cap tubes, respectively for later analysis in a certified human laboratory in the city. The samples for D-dimer were centrifuged immediately until the plasma was obtained. On the other hand, the blood count was evaluated in an automatic analyzer (Mindray BC-2800Vet) in a purple cap tube. The measurement ranges for cTnI were <0.02 ng ml^{-1} (Oyama et al 2004), for D-dimer <250 ng ml^{-1}, where there was evidence of a specificity of 90% and a sensitivity of 80% (Epstein et al 2013) and for the CRP <12 mg L^{-1} (Méndez et al 2014). The values below these were taken as negative and the values above as positive.

Statistical analysis

The data collected were tabulated through office Excel 2007, using InfoStat (version 2008 for Windows) for later analysis. It was used descriptive statistics (Mean, proportion and range), and the test of difference between two proportions. The confidence intervals for the different hematological variables, D-dimer tests, CRP and cTnI were estimated between the patients.

Results

Table 1 shows the percentages and confidence intervals (95%) of the clinical signs that had the patients studied. Highlighting the proportion of depressed individuals with a range from 66 to 97%, followed by dyspnea with ranges from 52 to 90%. Pulmonary hypertension was diagnosticated in 34 to 77% of the population, while dehydration was presented in 23 to 66%. Patients with microfilaraemia had a range from 47 to 87%.

Table 1. Average values of the clinical signs that had patients infected with *D. immitis*

Clinical signs	Percentage	CI (95%)
Fever	35	(16.0- 57.0)
Decay	87	(66.0-97.0)
Dehydration	43	(23.0-66.0)
Cough	30	(13.0-53.0)
Icterus	17	(0.5-39.0)
Dyspnea	74	(52.0-90.0)
Pulmonary hepertension	57	(34.0-77.0)
Pruritus	26	(10.0-48.0)
Syncope	17	(0.5-39.0)
Microfilariae	70	(47.0-87.0)
Death	0.9	(0.1-28.0)

The confidence interval (95%) of the concentration of hematocrit was from 38.29 to 47.77%. Females had average of 39.24%, and males of 44.08%. Hemoglobin and the count of red blood cells had a reliability of 95%, being found within 12.19-5.43 and 15.14 g dl^{-1} -6.72 $x10^{12}$, respectively. With respect to the leukocyte values, the average was 15.22 $x10^9$ L^{-1}. In the differential leukocyte count it was observed an average of 11.46 $x10^9$ L^{-1} for neutrophils, 2.45 $x10^9$ L^{-1} to lymphocytes and 0.76 $x10^9$ L^{-1} for monocytes. Platelet count had a mean of 258.32 $x10^9$ L^{-1}. Females had an average of 286.36 $x10^9$ L^{-1} and males had an average of 250.53 x 10^9 L^{-1} in the platelet count (Table 2).

D-dimer levels were high in 73.9% of the cases, with a mean of 3,391.78 ng ml^{-1}. Of this increase, 69.6% of the individuals had microfilaremia. Plasma levels of D-dimer in males had a significantly higher mean compared to females (3,857.83 vs 1,714.0 ng ml^{-1}). The cTnI values were high in 21.7% of patients with a mean of 0.10 ng ml^{-1}. Males showed increases in serum levels with a mean of 0.13 ng ml^{-1}, whereas females remained within the normal 0.01 ng ml. Finally, the CRP values were high in 39.1% of the individuals; both females and males had increases in their values, with a mean of 47.4 mg ml^{-1} for males and 36.0 mg ml^{-1} for females (Table 3). The microfilaremic patients did not show changes compared to the amicrofilaremic individuals for the values of cTnI and CRP.

Discussion

Few studies have documented the usefulness of Dimer-D, cTnI and CRP for their use in daily clinic. These cardiopulmonary and inflammatory biomarkers are recently used in veterinary medicine for the diagnosis of cardiovascular diseases (Boswood et al 2009), and studied in patients with *D. immitis* (Carretón et al 2011) experimental mode with myocardial damage and adulticide treatments of patients infected with *D. immitis* (Carretón et al 2013b). The measures for the Dimer-D, CRP and cTnI as biomarkers in the diagnosis, prognosis and treatment of lesions caused by the parasite, indicated myocardial damage, TE and inflammation.

The heartworm provoke in the host lesions in the artery and pulmonary parenchyma, triggering PH and PT due to thrombus formation or fragments due to the death of the adult parasites (Bowman et al 2009). Patients who had heartworm developed in the initial stages respiratory signs and in the final phase congestive signs, vena cava syndrome or sudden death. Within the respiratory clinical signs it is described coughing as one of the first signs (McCall et al 2008); 13 to 53% of the patients had coughing, but there was dyspnea in a greater proportion of individuals with a range from 52 to 90%. Thromboembolism has a poor prognosis and is potentially fatal. The most frequent signs are tachypnea, dyspnea, and depression. Other signs may be hemoptysis, cyanosis, syncope, collapse and sudden death (Goggs et al 2017). The common signs that patients had were suggestive of PT in different degrees, but these signs are non-specific and inconsistent.

Table 2. Hematological average values studied in patients with heartworm disease

Hematological values	Average (CI)	Females n= 5 (CI)	Males n=18 (CI)
Hematocrit %	43.03 (38.29-47.77)	39.24 (21.0-50.0)	44.08 (17.09-65.40)
RBC x10^{12} L^{-1}	6.07 (5.43-6.72)	5.62 (3.10-7.10)	6.20 (2.50-8.70)
Hemoglobin g dL^{-1}	13.67 (2.19-15.14)	12.72 (7.0-16.60)	13.93 (5.60-18.70)
WBC x10^{9} L-1	15.22 (10.02-20.42)	17.22 (8.70-44.6)	14.67 (6.10-58.90)
Neutrophils x10^{9} L^{-1}	11.46 (6.41-16.52)	12.80 (5.10-39.90)	11.09 (0.59-53)
Lymphocytes x10^{9} L^{-1}	2.45 (1.85-3.04)	2.38 (0.69-3.90)	2.47 (0.62-5.20)
Monocites x10^{9} L^{-1}	0.76 (0.54-0.97)	1.28 (0.80- 2.10)	0.61 (0.18-1.40)
Platelets x10^{9} L^{-1}	258.32 (212.05-304.58)	286.36 (188-393)	250.53 (114-466)

CI: confidence interval, RBC: Red blood cells, WBC: White blood cells

Table 3. Mean values of cardiopulmonary and inflammatory biomarkers of dogs infected with the heartworm

Variable	Result	Males n=5	Females N=18
D-dimer ng ml^{-1}	3.391.78	3.857,83	1.714.0
Troponin I ng ml^{-1}	0.1	0.13	0.01
C-reactive protein mg L^{-1}	44.8	47.4	36

There are no findings of pathognomonic laboratories for the diagnosis of the heartworm, thromboembolism, or the severity of the disease that the patient is facing. The majority of dogs with heartworm disease have a normal blood count (Montoya et al 2012). The complete blood count does not discriminate the occurrence of PT (Goggs et al 2017). In hematological alterations during the formation of thrombi, the thrombocytopenia, anemia and leukocytosis are reported and leukopenia is the least frequent (Respess et al 2012). In our study the hematocrit remained within normal limits (38.29 - 47.77%). Females showed a red blood cell count that was lower compared to the males.

Platelet count can be normal or high despite the formation of clots that could cause platelet consumption in patients infected with *D. immitis* as reported by Lynelle et al in 1999 (thrombocytopenia in 40% of cases). In our study, it remained within the reference values in the range from 212 to 304 x109 L^{-1} despite the D-dimer plasma increases. In addition, a range of leukocytes was observed from 10.02 to 20.42 x109 L^{-1} with a tendency to leukocytosis where males showed higher increase compared to females. The increase is suggestive for pulmonary inflammation generated by parasites at this level or by PT. The leukocyte increase as a risk factor has been reported in canine patients (Thawley et al 2016) and human patients (Buxhofer-Ausch et al 2012). 30% of the patients did not present microfilaremia, suggestive of the age of the parasites or the immune response to microfilariae or adult parasites (McCall et al 2008). Arterial endothelial alteration and reduction of the pulmonary arterial diameter due to thrombus or to the presence of adult parasites, together with inflammatory mediators, can lead to the presentation of PH (Simón et al 2012). This pathology was reported from 68% (Serrano et al 2017a) to 70.4% (62-88%) (Serrano et al 2017b). A similar finding was observed in 57% (34-77%) of the studied population which was considered a frequent and severe phenomenon of the disease; it was present at the time of the diagnosis and it persisted 120 days after the application of adulticide therapy (Serrano et al 2017a).

The classic triad of signs of PT in humans include dsypnea, pain in the chest and hemoptysis; dsypnea occurs around from 59 to 90% (Lynelle et al 1999) and 90% (Klein et al 1989) of the cases. Data that is similar to the reported information were found in our study in ranges from 52 to 90%. Dimer-D is a fibrin degradation product; high plasma concentrations indicate the presence of thrombi or their degradation. This test is more sensitive and specific as opposed to thromboelastography.

Studies have found no significant differences between, but several experimental approaches did find a correlation between the presence of thrombi and an increase of Dimer-D (Thawley et al 2016; Lennon et al 2013). In addition, it was reported that dogs naturally and experimentally infected with *D. immitis* have deposits of Dimer-D in 34.8% to 40% of pulmonary tissue (Carretón et al 2013a), and 47% in heartworm-infected patients (Carretón et al 2011). In our investigation there were deposits of Dimer-D in 73.9% of pulmonary tissue.

Thrombosis is more common in females than in males (Thawley et al 2016; Stokol et al 2003). The relation of coagulation to sex has been identified using thromboelastography in humans in healthy individuals (Gorton et al 2000) and trauma patients (Schreiber et al 2005), possibly caused by the increase in fibrinogen, factor III and low concentrations of S protein that would explain the tendency to thrombosis in people (Lowe et al 1997). The hormonal role was not fully elucidated, but females were more likely even if they are sterile (Thawley et al 2016). Epidemiological studies of the coagulation factors have not been reported in dogs or if females or males produce more thrombi, but in this study it was found that males (3,857.83 ng ml^{-1}) had an average higher with respect to females (1,714.0 ng ml^{-1}).

The cTnI is a sensitive and specific biomarker of myocardial injury which has been used as a diagnosis in various cardiac pathologies in dogs (Oyama et al 2004). Slight increases in cTnI were found in dogs with circulating, microfilaremic and amicrofilaremic antigens in infected individuals (Carretón et al 2011). 21.7% of evaluated patients showed increases of the cTnI. Males had higher values compared to females. It was that the increase of cTnI is caused by the PH and consequently due to the right heart failure (Carretón et al 2012), but the analysis of the data did not showed any relation between the increase of cTnI and

the presence of PH. Similar results were reported by Carretón et al (2012) where they correlated microscopic findings and myocardial with staining anti-cTnI antibodies. Meanwhile, the levels of Dimer-D and CRP did increase in PH, which suggests that the presence of thrombi and inflammation can develop this pathology (Ben et al 2007).

Conclusions

This retrospective study reaffirmed the effectiveness of the use of biomarkers in predicting the evolution of dogs with *D-Immitis* disease through the mean hematological values showed in Table 2 and the average cardiopulmonary and inflammatory values in Table 3. However, unlike other similar experiments, our study reported a greater coverage of Dimer-D in lung tissues of 73.9% On the other hand, despite epidemiological studies of the coagulation factors have not been reported in dogs or if females or males produce more thrombi, this study found that males had an average higher compared to females. Both results opens the door for future trials and analysis under similar conditions. The analysis of the data did not showed correlation between cTnI and the presence of PH.

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