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Detection and laboratory findings due to *Ehrlichia canis* in dogs from the south area of São Paulo – SP, Brazil

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ABSTRACT

OBJECTIVE

To evaluate hematological changes in dogs diagnosed with *Ehrlichia canis*.

METHODS

We analyzed 54 dogs seen at the Veterinary Hospital of Universidade Santo Amaro, São Paulo/SP, Brazil. During the appointment, rectal temperature was measured, and venous blood samples were collected for blood count and serological and molecular testing by indirect immunofluorescence and real-time polymerase chain reaction (PCR), respectively.

RESULTS

Among the 54 animals suspected of having *E. canis*, 32 (59.3%) were diagnosed as positive. Of these, 23 (42.6%) were positive in the serological test alone, while 9 (16.7%) were positive in both serological and molecular tests. Thrombocytopenia was the most important alteration found in this study and was the only parameter found in the blood count that presented statistical difference.

CONCLUSIONS

Thrombocytopenia was the main finding in infected animals and that the combination of molecular and serological diagnostics may increase the chances of detecting infection or exposure to the agent.

DESCRIPTORS

Canine Monocytic Erlichiosis, Tick, Thrombocytopenia, Anemia.

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INTRODUCTION

Canine Monocytic Ehrlichiosis (CME) is a disease transmitted in Brazil by the tick *Rhipicephalus sanguineus*, whose main host is the dog[1].

The presentation of the disease in the dog can vary from mild to severe, depending on the immunological conditions of the host, the virulence of the isolate and the co-infection with other microorganisms[2,3,4]. Laboratory support is required for confirmation of infection[5]. The most used tests are Polymerase Chain Reaction (PCR) and indirect immunofluorescence, but the positive and/or negative predictive value varies according to the stage of infection in the animal[6,7,8,9,10].

The Hospital Veterinário da Universidade Santo Amaro (HOVET-UNISA), located in the south of the city of São Paulo/SP, Brazil, has a high number of cases of canine ehrlichiosis, so the objective of this study was to evaluate the hematological changes in dogs with molecular diagnosis. and/or serological testing for *Ehrlichia canis* treated at this site.

METHODS

This study evaluated 54 (fifty-four) male and female dogs, of different ages and breeds, that had been treated at the Veterinary Hospital of the Santo Amaro University, located in the South Zone of São Paulo. Convenience sampling was used among dogs that had presumptive diagnoses of Canine Monocytic Ehrlichiosis (CME), since they presented clinical signs reported in the literature as being compatible with the disease, such as anorexia, hyporexia, apathy, diarrhea, hyperthermia, emesis, lymphadenopathy, petechiae, neuropathies and ocular alterations[11]. This study was approved by the Ethics Committee on the Use of Animals of the Santo Amaro University (CEUA-UNISA), with opinion number: 16/2018.

During the consultation, the animals' rectal temperature was measured, and venous blood samples were collected for hemogram, serological and molecular tests. Hematocrit, hemoglobin, red blood cells, leukocytes and platelets values were obtained in an automated analyzer. The differential leukocyte count was performed according to Rosenfeld[12]. The hematological parameters reported by Feldman et al.[13] were used as reference values.

Blood samples were individually processed for DNA extraction using the Purelink Genomic DNA Kit by Invitrogen®.

The material obtained by canine blood extraction was analyzed by polymerase chain reaction (PCR) with the intention of amplifying the partial sequence of nucleotides of the dsb gene, through the use of initial oligonucleotides called Dsb-321 (5'- TTGCAAAAGAAGAAGATATGAAACA – 3'), Dsb-671 (5' - GCTGCTCCACCAAAATATATCYCCTA – 3'), and the TaqMan probe (5'- AGCTAGTGCTGCTTGGGCAACTTTGAGTGAA – 3') 5' FAM/BHQ – 13', specific for *E. canis* species, as previously standardized[14]. Amplification, acquisition and data analysis were performed on a multicolor detection system for Real Time PCR (7500 Real Time PCR Systems – Applied BioSystems, Foster City, CA, USA).

Serum samples from the animals were submitted to the Indirect Immunofluorescence (IFN) reaction for detection and titration of anti-*E. canis*, according to Aguiar et al.[15]. The plates for RIFI had already been sensitized with DH82 cells infected by the isolate Cuiabá#1. Sera that showed fluorescence intensity at a dilution greater than 1:40[7] were considered positive.

The results were analysed using the computer program Statistical Analysis System[16], and the normality of the residuals was previously verified by the SHAPIRO-WILK Test (PROC UNIVARIATE). Data were submitted to analysis of variance using the method of least squares using the PROC GLM procedure of the Statistical Analysis System[17] computer program. A significance level of 5% was adopted for all tests performed and in the analysis of variance, when a significant effect was observed, a comparison of the means was performed using the Tukey test.

RESULTS

Of the 54 dogs suspected of *E. canis*, 32 (59.3%) were diagnosed as positive. Of these, 23 (42.6%) were positive only in the serological test (hereinafter referred to as group 1), while 9 (16.7%) were positive in both molecular and serological tests (referred to as group 2 from now on).

The positive samples for *E. canis* in the serological and molecular tests showed serological titers ranging between 2,560 and 10,240. The mean age of these animals was 5.74 years. The mean rectal temperature was within the normal range (38.6°C) and none of the animals had hypothermia at the time of measurement. As for leukocytes, 7/9 (77.8%) of the positive animals in both tests had leukocytes within the normal range, maintaining an average of 15,200 thousand/mm³, with only 2/32 (6.2%) showing leukocytosis, with a maximum value of 43,500 thousand/mm³. The total average of platelets was 77,667 thousand/mm³, and only 1/32 (3.1%) animal presented a value (205 thousand/mm³) within the normal range; and the animal with the most severe case of thrombocytopenia had 18,000 thousand/mm³. Regarding the values found for erythrocytes, only one animal presented total values of red blood cells (6.4 million/mm³) above the lower reference limit; the others had low levels of anemia. For example, the lowest red blood cell count was 1.4 million/mm³, and the average was 4.34 million/mm³. Hemoglobin and hematocrit values averaged 9.57 million/m³ and 28.6% respectively (Table 1).

Table 1. Values of blood count, age, and temperature of dogs presenting clinical alterations with molecular and serological diagnosis for *E. canis* and admitted to the Veterinary hospital in the south of São Paulo/SP in 2018.

Animal No.	Age years	Temperature °C	Leukocytes cel./mm³	Platelets mil/mm³	Red blood cells million/mm³	Hemoglobin g/dl	Ht %	PT g/dl
3	5	38.1	5,100	50,000	4.39	10	30	7.2
6	1	38.7	14,000	41,000	6.4	15.1	45	6.2
11	9	38.1	33,200	59,000	1.4	3.9	11	5.6
16	10	38.6	7,800	70,000	5.26	11.5	32	8.4
27	8	38.7	5,200	205,000	4.45	9.3	28	11.6
31	9	38.6	14,600	18,000	3.3	6.9	19	3.6
47	0,5	39	5,100	55,000	4.89	10.4	36	6.8
49	4	39.2	43,500	132,000	5.3	10.9	32	6
52	5	38	6,400	69,000	3.7	8.2	24	6

The 23 (42.6%) animals that were positive only in the serological test had titers ranging between 40 and 10,240. The average age of these animals was 6.43 years, with an average temperature within the normal range (38.7°C); however, four animals had hyperthermia at the time of measurement. As for leukocytes, 4/23 (17.4%) of the dogs had leukopenia; 23/8 (34.8%), leukocytosis; and 23/11 (47.8%) were within the normal range. Of all the dogs tested, an average of 13,020 thousand/mm³ was maintained, with a maximum value of 29,200 thousand/mm³ and a minimum of 400 thousand/mm³ leukocytes. The total average of platelets was 149,520 thousand/mm³, with only 6/23 (26%) animals presenting platelet counts within the normal range. The animal with the most severe thrombocytopenia had 13 mil/mm³. 30.4% (23/7) of the animals had anemia, with an average red blood cell count of 5.41 million/mm³, with the highest and lowest red blood cell counts being 0.8 million/mm³ and 8 million/mm³, respectively. Hemoglobin and hematocrit values averaged 12.42 million/mm³ and 36.4% respectively (Table 2).

Table 2. Values of blood count, age, and temperature of dogs presenting clinical alterations with positive serological diagnosis for *E. canis* admitted to the Veterinary hospital in the south of São Paulo/SP in 2018.

Animal No.	Age years	T emperature ºC	Leukocytes cel./mm³	Platelets thousand/ mm³	Red blood cells million/mm³	Hemoglobin g/dl	HTO %	PT g/dl
1	6	39	18,300	90,000	5.5	13.2	37	6.6
2	8	39.6	9,500	70,000	5.9	14.2	40	5.6
4	-	36.6	18,500	237,000	5	11.4	36	12
7	10	39	8,200	35,000	4.67	9.7	32	7.4
9	4	38.4	10,700	66,000	3	5.9	19	6.2
13	9	37.7	12,500	92,000	5.4	12.1	34	12
14	13	39.1	4,700	350,000	7.4	18.7	53	7
17	15	37.9	12,700	83,000	7.1	16.7	49	9.2
22	4	39	5,300	53,000	5.5	13.3	40	7.2
23	5	39.3	19,600	377,000	7.1	17.7	49	7.6
24	2	37.9	21,780	492,000	4.63	8.7	24,8	6.8
26	1	38.7	22,200	100,000	3.1	7.3	22	5.8
28	6	39.3	7,700	131,000	1.7	3.3	12	6.8
29	4	40.4	29,200	166,000	8	18.3	53	8.8
33	1	39.9	400	15,000	0.8	1.9	6	4.4
36	3	38.7	16,000	255,000	7.4	17.9	50	7.2
38	4	38.5	8,000	144,000	7.8	19	53	5.4
40	8	38.2	6,600	60,000	6.9	17.7	49	6
44	9	39.3	16,100	142,000	6.99	15.4	48	7.6
46	-	39.9	3,100	13,000	5.84	10.7	33	6.2
48	13	39.1	19,200	99,000	3.9	8.2	24	8.6
50	4	37.5	9,700	161,000	5.8	13.6	41	12
51	6	38.2	19,900	208,000	5.03	10.8	32	10.4

From the statistical analysis of the parameters evaluated and presented in table 3, it is possible to confirm a significant difference between the platelet values of the animals with or without a diagnosis of MSC (p=0.0245). As for the values of other parameters of red blood cells and rectal temperature, no statistical differences were observed (Table 3).

Table 3. Average values and standard deviations from the parameters of hemogram and rectal temperature of patients with presumptive diagnosis for CME admitted to the Veterinary hospital in the south of São Paulo/SP in 2018.

	Positive in both molecular and serological tests		Positive only in serological test		Negative in both molecular and serological tests		p=
	Average	CV%	Average	CV%	Average	CV%	
Temperature	38.55^{a} ± 0.41	1.07	38.69^{a} ± 0.77	2.00	38.30^{a} ± 1.36	3.56	0.3897
Hematids	4.34^{a} ± 1.43	33.02	5.41^{a} ± 1.92	35.51	4.87^{a} ± 2.10	43.19	0.3454
Hb	9.57^{a} ± 3.12	32.63	12.42^{a} ± 4.97	40.05	11.42^{a} ± 4.83	42.33	0.3070
Hto %	28.55^{a} ± 9.82	34.40	36.39^{a} ± 13.55	37.23	33.04^{a} ± 13.90	42.08	0.3124
Pt	6.82^{a} ± 2.21	32.39	7.68^{a} ± 2.15	28.08	6.75^{a} ± 1.63	24.16	0.2529
Platelets x10³	77.66^{c} ± 56.82	73.16	149.52^{b} ± 122.29	81.78	251.19^{a} ± 228.45	90.94	0.0245
Leukocytes x10³	15.2^{a} ± 13.97	93.23	13.02^{a} ± 7.25	55.69	19.25^{a} ± 13.91	72.29	0.2003

DISCUSSION

Thrombocytopenia was the most important statistical factor found in this study, showing different degrees of intensity, being present in 88% (8/9) of patients tested positive for *E. canis* with molecular and serological diagnosis; 73% (11/15) in dogs tested positive with only serological diagnosis; and 45% (22/10) of those tested negative with the diagnostic methods used in this study.

In a study carried out with the objective of verifying the reliability of the platelet count as a screening test for CME, 63% (84/146) of thrombocytopenic animals were positive for *E. canis*[2]. Thrombocytopenia has also been described in other studies involving animals with the disease[18,19,20].

Thrombocytopenia is the most common finding in all three stages of the disease and can occur due to consumption losses in the case of vasculitis, immune-mediated destruction and/or sequestration of platelets in the spleen caused by stimulation of the immune system, and in part, due to the inflammatory response[21,22].

The pathogenesis of thrombocytopenia in dogs caused by CME remains poorly understood, but it is believed that it may be associated with changes in the endothelium of blood vessels and by disseminated intravascular coagulation[20,21,23]. Weisiger et al.[24] reported that the immune response of dogs to *E. canis* can potentiate the pathogenesis of the disease, based on the presence of hypergammaglobulinemia and systemic plasmacytosis[24]. Studies show that serum antiplatelet antibodies in dogs after *E. canis* infection is possibly one of the immunopathological causes responsible for thrombocytopenia[25], in which the decline in the number of platelets can be explained by the premature appearance of this immune response, causing the removal of platelet cells by the system. mononuclear phagocytic cells in the liver and spleen[25,26]. The presence of antiplatelet antibodies is proposed to be a major cause of thrombocytopenia seen in CME, although other non-immunological diseases and mediated mechanisms may also be involved[26].

All the hematological findings found, even if present in CME, do not exclusively point to this type of disease, as they are nonspecific and inconstant, they can also be indicative of other diseases, as well as transient physiological changes related to the subject's life stage[27].

Anemia can be found in both acute and chronic phases of the disease[28]. In the present study, this hematological alteration was present in animals that presented molecular and serological positivity for the bacteria, something also found in a study carried out in Minas Gerais with 203 animals diagnosed with *E. canis*, in which 82.3% presented this hematological alteration[27,28].

According to Hasegawa[29], leukopenia is common in the terminal stages of the disease, rarely in its acute phase. This finding has been present not only in animals that tested positive in the molecular and serological diagnosis, but also in those that tested negative for *E. canis*. This suggests that when leukopenia is present, the disease should be considered a hypothesis; but other causes, such as the normal transient variation related to the subject's life stage, inflammatory, parasitic, toxic and anaphylactic causes cannot be ignored[27]; nor viral diseases and neoplasms[21,30].

The serological method is not able to distinguish a present infection from an exposure to the agent, since the titer remains high for some time[11]. The presence of positive serology and negative PCR may indicate a present immunological memory or a situation in which the animal is undergoing treatment, since antibiotics eliminate the microorganisms circulating in the bloodstream[11]. However, the negative molecular diagnosis, although seropositive, may suggest that the bacteria may be present in other tissues, such as the spleen and bone marrow, undetectable in peripheral blood samples[6,10,11]. According to Harrus et al.[9], the molecular detection of *E. canis* obtained from peripheral blood is inferior when compared to that found in the spleen or marrow; causing false negatives to occur in chronic infections of the disease, because at this stage of the disease, bacteremia in the peripheral bloodstream is scarce[31]. In the present study, 23 (42.6%) animals were positive only in the serological diagnosis, suggesting in these patients the possibility of having chronic infections. Molecular research at the tissue level is recommended[31].

Serology and PCR are the most appropriate tests to confirm the diagnosis of canine monocytic ehrlichiosis; however, they should always be used in conjunction with clinical and hema-

tological assessments. For a better interpretation of laboratory results, it is important to take into account the stage of infection, limitations of these tests and epidemiological data from the area under study. In the acute phase, PCR can detect *E. canis* DNA before serological tests come into contact with the presence of antibodies against the etiologic agent. In addition, cross-reactivity of molecular tests is uncommon, although false positives may occur on serology due to cross-reaction with other species or antibody titers that are persistent after treatment[32].

The high serological titer values found in this study have already been mentioned in the literature, as in Frank et al.[18]. Most animals had antibody titers between 2,560 and 10,240, suggesting recent or persistent infection possibly related to the chronicity of the disease and exceptional type of antigen response[7], originated by prolonged antigenic stimuli[33].

CONCLUSION

This study concludes that thrombocytopenia was the only hematological parameter that presented a statistical difference in the analysed groups and can be considered an important laboratory data in conjunction with molecular and serological tests for the detection of the disease.

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