Hematologic Parameters in Polymerase Chain Reaction-Positive and -Negative Dogs for *Anaplasma platys* Presenting Platelet Inclusion Bodies

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ABSTRACT

Cyclic thrombocytopenia is a rickettsial-disorder caused by *Anaplasma platys*, which belongs to the family Anaplasmataceae, genus *Anaplasma*. It was first described in 1978 in Giemsa-stained blood smears as basophilic inclusion bodies in platelets from thrombocytopenic dogs and has been incriminated in causing thrombocytopenia in dogs from Brazil. The most common laboratory findings are thrombocytopenia, anemia, macroplatelets, monocytosis, and hypoalbuminemia. Although thrombocytopenia is a common finding associated with *A platys* infection, this finding is not pathognomonic of this rickettsiosis or any other disease. The aim of this study was to describe hematologic findings, with emphasis in thrombocytopenia, from 101 dogs presenting platelet inclusion bodies resembling *A platys* or not. All 101 samples were submitted to polymerase chain reaction (PCR), carried out in 2 stages, with *A platys* infection confirmed by this method in order to provide a better interpretation of laboratory data. Using Student's t-test, it was concluded that the PCR1-postive and the PCR2-positive groups behaved statistically the same for all hematologic parameters, but when comparing negative and positive groups, there was a significant difference for red blood cell count, hemoglobin concentration, packed cell volume, and platelet count. Although 100% of *A platys*-positive animals presented with thrombocytopenia (some with anemia), the physician should include other diseases (besides *A platys*), with similar laboratory data, in differential diagnosis.

INTRODUCTION

Anaplasma platys is an obligatory intracellular microorganism that acts as a parasite

exclusively in canine platelets[1,2,3] and presents with typical morphologic and behavior characteristics. Cyclic thrombocytopenia is a rickettsial-disorder caused by this parasite that belongs to the family Anaplasmataceae, genus *Anaplasma*.[4] It was first described in 1978 as *Ehrlichia platys* in Giemsa-stained blood smears,[5] as basophilic inclusion bodies in platelets from thrombocytopenic dogs, and has been incriminated in causing thrombocytopenia in dogs from Brazil.[6]

The probable vector of *A platys* is the tick Rhipicephalus sanguineus,[7,8] as other agents such as *Ehrlichia canis* and Babesia canis are also commonly transmitted by this same invertebrate. The possibility of simultaneous infections cannot be discarded and would produce a worsening of clinical disease.[9,11]

Animals with parasites will present the first symptoms 8 to 15 days after *A platys* infection. During acute phases of the disease, a high percentage of infected circulating platelets can be found. After that, a decrease of circulating platelets is common, leading to counts as low as 20,000/μL, making it difficult to visualize the parasite. After the disappearance of the microorganisms, the platelet counts come back to normal in 3 to 4 days. In 7 to 14 days, another parasitemia occurs when, again, the number of platelets decreases. This cyclical nature of thrombocytopenia and the parasitemia diminishes with time as the disease enters its chronic phase, which may result in sporadic appearances of the parasite and mild to moderate thrombocytopenia.[1,12-14]

Although thrombocytopenia is a common finding associated with *A platys* infection, this is not pathognomonic of this rickettsiosis or any other disease,[15] but may include *A platys* and *E canis* as differential diagnosis.[16] Immune-mediated disorders, neoplasia, hypoproliferative bone marrow, various inflammatory diseases, trauma, acute bleeding, disseminated intravascular coagulation,[17] and splenic or hepatic sequester[18] also cause reduced platelet counts. Other common causes are viral, bacterial, protozoan and even other rickettsial infections.[19]

The most common laboratory findings are thrombocytopenia, anemia,[16] macroplatelets, and monocytosis.[20,21] The nonspecific laboratory data make it risky to approach the diagnosis with only complete blood count (CBC) analysis. The presence of platelet inclusion bodies in thrombocytopenic dogs coupled to the indirect immunofluorescence results can mislead the clinician to a positive diagnosis.[20]

The aim of this study was to describe hematologic findings, with emphasis on thrombocytopenia, from 101 dogs presenting platelet inclusion bodies resembling *A platys* or not. All 101 samples were submitted to polymerase chain reaction (PCR), with *A platys* infection confirmed by this method in order to provide a better interpretation of laboratory data and diagnostic approach to the physician.

MATERIAL AND METHODS

The number of dogs sampled was determined after the minimum sample size of 96 dogs was established with an estimated error of less than 10%, supposing a prevalence of 50% in an infinite population.[22] Platelet inclusion bodies resembling *A platys*[2] or not were identified in stained blood smears under light microscopy from 101 EDTA-blood samples during CBC performed under an automation system using a Coulter T-890. The dogs were males and females of different breeds and ages, from different localities of Rio de Janeiro city, Brazil.

The samples containing platelet inclusion bodies were kept at -19°C and sent to the Laboratório de Bactérias Enteropatogênicas e Microbiologia de Alimentos, Instituto Biomédico (Universidade Federal Fluminense) for PCR testing.

The PCR was carried out in 2 stages: the first was done to select some members of Anaplasmataceae family previous described in Rio de Janeiro, Brazil, and only the positive ones were tested in a second protocol specific for *A platys*.

Three controls were used in this research aiming to access PCR efficiency and possible contamination. The positive control consisted of a DNA solution extracted from

a blood sample from a dog known to be infected by *A platys*. This positive control was tested in Universidade Estadual Paulista–Jaboticabal first for the species *E canis* (proving negative), then tested for genus *Anaplasma* (being positive), then tested for *A phagocytophilum* (being negative), and finally showed positive for *A platys*. To confirm the PCR positivity to *A platys*, the products were used for DNA sequencing. The negative control consisted of a DNA solution from *Escherichia coli* strain K12 DH5α23 extracted by the boiling method. The control with no DNA consisted of a mixture of reagents without the final addition of DNA.

DNA extraction of the 101 samples was performed using GFX™ kit (Genomic Blood Purification Kit, Amersham Biosciences, Piscataway, New Jersey, USA) according to the manufacturer instructions.

PCR1 Protocol

First, the samples were tested for the amplification of a 345-bp fragment from 16S ribosomal RNA common to several species of the family Anaplasmataceae: *E canis*, *Ehrlichia chaffeensis*, *Ehrlichia muris*, *Ehrlichia ruminantium*, *A phagocytophilum*, *A platys*, *Anaplasma marginale*, *Anaplasma centrale*, *Wolbachia pipientis*, *Neorickettsia sennetsu*, *Neorickettsia risticii*, and *Neorickettsia helminthoeca*.[7,24] This analysis (PCR1) used the forward primer EHR16SD (5' - GGT ACC YAC AGA AGA AGT CC - 3') and the reverse primer EHR16SR (5' - TAG CAC TCA TCG TTT ACA GC - 3') as the protocol described in 2001.[24,25]

PCR2 Protocol

The positive PCR1 DNA samples were tested again in a second protocol, specific for *A platys* (PCR2) using forward primer PLATYS (5' - GAT TTT TGT CGT AGC TTG CTA TG - 3') combined with reverse primer EHR16SR (5' - TAG CAC TCA TCG TTT ACA GC - 3'), which amplifies a 678-bp fragment from 16S ribosomal RNA.[24-26]

The amplification reactions for *A platys* were done using a similar protocol, but the forward primer EHR16SD was exchanged by PLATYS combined with the reverse primer EHR16SR.[24-26]

The amplification products were added to 1% agarose gel (Amersham Biosciences, Piscataway, New Jersey, USA) and placed in a horizontal electrophoresis chamber for approximately 40 minutes at 125 volts (CBS - MGV502 T, CBS Scientific Company Inc., Del Mar, California, USA).

The data were analyzed using SPSS software v.10.0 (SPSS Inc, Chicago, Illinois, USA). Student's t-test and analysis of variance (ANOVA) were done to compare the differences in hematologic parameters between the positive and negative animals by PCR method.

RESULTS

Table 1 summarizes the hematologic findings from the positive and negative groups. The ANOVA was applied to check if hematologic parameters behaved in the same manner in all 3 groups (PCR1-positive; PCR2-positive; and negative).

For mean corpuscular volume (P = 0.350), mean corpuscular hemoglobin concentration (P = 0.908), and white blood cell count (P = 0.955), there was no significant difference (with a significance level of 0.05).

For red blood cell count (P = 0.00), hemoglobin concentration (P = 0.00), packed cell volume (P = 0.00), and platelet count (P = 0.00) there were significant differences.

Using Student's t-test, we concluded that the PCR1-postive (n = 19) and the PCR2-positive (n = 16) groups behaved statistically the same for all tested parameters (P = 0.911 for red blood cell count, P = 0.787 for hemoglobin concentration, P = 0.831 for packed cell volume, and P = 0.965 for platelet count).

Comparing negative (n = 82) and positive groups, using Student's t-test, we concluded that there is significant difference for red blood cell count (P = 0.00), hemoglobin concentration (P = 0.00), packed cell volume (P = 0.00), and platelet count (P = 0.00).

Table 1: Mean values of hematologic parameters from dogs presenting inclusion bodies in platelets, tested by PCR to Anaplasmataceae family (PCR1) and A platys (PCR2), divided by PCR results.

Parameters	N	Mean Value	SD
PCR1			
PCV (%)	19	29.0	7.0
RBC (×10³/µL)	19	4.2	1.0
HGB (g/dL)	19	9.5	2.4
MCV (fl)	19	69.0	4.0
MCHC (%)	19	33.0	1.6
WBC (/µL)	19	11,337	6,212
Platelet count (/µL)	19	59,895	50,775
PCR2			
PCV (%)	16	29.0	7.0
RBC (×10³/µL)	16	4.2	1.0
HGB (g/dL)	16	9.7	2.5
MCV (fl)	16	69.0	4.0
MCHC (%)	16	33.2	1.6
WBC (/µL)	16	10,894	6,282
Platelet count (/µL)	16	59,125	51,226
Negatives			
PCV (%)	82	40.0	10.0
RBC (×10³/µL)	82	5.9	1.6
HGB (g/dL)	82	13.4	3.4
MCV (fl)	82	67.9	4.3
MCHC (%)	82	33.2	1.7
WBC (/µL)	82	11,657	10,586
Platelet count (/µL)	82	249,146	149,924

PCV = packed cell volume; RBC = red blood cells; HGB = hemoglobin; MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration; WBC = white blood cells.

DISCUSSION

The mean platelet count for Anaplasmataceae positive animals (PCR1) was below the low end reference value.[27] This is in accordance with many authors who have reported an association between thrombocytopenia and the species covered by the primer sequences used.[1,2,5,13,18,20,27-31]

The thrombocytopenia is caused by the parasite proliferation,[1,2,16] which after several cycles, turns into an immunomediated mechanism.[14] This suggests the thrombocytopenia may be an indicator of *A platys* infection and that this agent must be taken into consideration during thrombocytopenia differential diagnosis,[16,32] as 100% of the positive samples to *A platys* were thrombocytopenic (Table 1). The mean platelet count from the negative animals ranged from normal to low values. These low values might have happened due to other thrombocytopenia causes.[17,18,21] One of the animals was PCR1-positive (family Anaplasmataceae), PCR2-positive (*A platys*) and, through blood smear evaluation, Babesia sp.-positive, confirming the possibility of the presence of 2 different parasites.[33] This animal presented with intense thrombocytopenia, justified by the co-infection that potentializes the effect, making diagnosis and therapy more difficult.[11]

The mean packed cell volume for the PCR2-positive (*A platys*) animals (Table 1) was below the low end reference value[27] and was considered anemic. Mild anemia may occur during clinical disease and is classified as normocytic/normocromic, similar to inflammatory disease anemia.[9,33,34] The latter is multifactorial and mediated by cytokines secreted during inflammation.[34] The occurrence of positive animals with other types of anemia can be justified by co-infections and variation in nutritional status.[1,2,20,35-39]

The mean white blood cell count was within the reference values range.[27] In *A platys* infections, it is common for the white blood cell count to remain unaltered.[1,2,13] When analyzing individual results, however, some infected animals presented a mildly reduced white blood cell count as observed in other studies,[1,34,40] while other animals were severely leukopenic. This was justified by a possible co-infection with *E canis*,[14,33,41]

which causes a severe leukopenia due to a hypoproliferative bone marrow[1,13] or other parasites.[29] Further analysis such as serological investigation must complete this research.

CONCLUSION

The low platelet count in all *A platys*-positive animals of this paper make it important to include *A platys* infection as differential diagnosis in thrombocytopenic dogs. The diagnosis panel should also include other diseases with similar laboratory data.

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