Anaplasma platys Diagnosis in Dogs: Comparison Between Morphological and Molecular Tests

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ABSTRACT

Anaplasma platys is related to the appearance of inclusion bodies in blood platelets; however, this may be a nonspecific occurrence as there are nonparasitic inclusion bodies within these figured elements. Aiming to validate the morphological diagnosis for *A platys*, 101 dogs were selected due to the appearance of inclusion bodies, independently from suggestive parasites, which were submitted to polymerase chain reaction (PCR) carried out in 2 stages. The first stage consisted of the utilization of initialing sequences or specific primers for the detection of some species of the *Anaplasmataceae* family, such as: *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia muris*, *Ehrlichia ruminantium*, *Anaplasma phagocytophilum*, *A platys*, *Anaplasma marginale*, *Anaplasma centrale*, *Wolbachia pipientis*, *Neorickettsia sennetsu*, *Neorickettsia risticii*, and *Neorickettsia helminthoeca* (PCR1). The second stage consisted of the utilization of specific primers for the detection of the species *A platys* (PCR2). Upon comparison of the results, 18.81% of the studied animals showed positive for PCR1. For PCR2, 15.84% of the studied animals had a positive result. In the morphological analysis of the inclusion bodies, 14.85% of the animals showed positive for *A platys*. The other inclusion bodies were considered as nonspecific, therefore negative. When compared to the morphological analysis, the results of the molecule analysis by means of the MacNemar test led to the conclusion that there was no significant difference between the tests, which indicates that blood smear analysis is a good alternative to *A platys* diagnosis. The possibility of PCR use has not been discarded, as this is a highly specific test. The chance of a false-negative among the PCR-negative animals exists, since a reduced quantity of microorganisms may cause inflammation and the appearance of nonspecific inclusion bodies

that may not be sufficient for a detectable enlargement.

INTRODUCTION

Cyclic thrombocytopenia is a rickettsial-disorder caused by *Anaplasma platys* that belongs to the family Anaplasmataceae, genus *Anaplasma*.[1] It was first described in 1978 in Giemsa-stained blood smears, as basophilic inclusion bodies in platelets from thrombocytopenic dogs.[2]

The probable vector of *A platys* is the tick *Rhipicephalus sanguineus*.[3,4] Other agents such as *Ehrlichia canis* and *Babesia canis* are also commonly transmitted by the same invertebrate.[5-7]

Among the existent diagnostic methods, the most commonly used include: morulae identification in blood smears; antibody detection by indirect immunofluorescence; or DNA amplification by polymerase chain reaction (PCR).[8]

Due to the cyclic nature of this disease, seeing the microorganism inside the platelets of diseased animals is not an easy task,[3] and is usually an accidental finding.[9] This can be explained by a decreased platelet count during infection and, consequently, decreased circulating microorganism numbers. This low frequency of parasites in the blood smear makes the method imprecise, especially during thrombocytopenic phases.[2,10-12] In order to find the morulae, a careful and extensive analysis of the blood smear must be performed under light microscopy.[13] *E canis* infections can render inclusion bodies in some blood cells, including platelets, at some stage[14,15] and the possibility of granules visualization due to platelet activation can neither be discarded. These inclusions can be misidentified as *A platys* morulae.[15]

Finding inclusion bodies in stained blood smears is not a completely trustable method for the diagnosis of *A platys* infection, and the serological tests can render either a false-positive or -negative result as the presence of anti-*A platys* antibodies does not mean clinical infection but rather exposure to the agent.[16-18] Therefore, using PCR as a diagnostic tool with its high sensitivity, specificity, and quick results[15] may provide for a better diagnostic test.

The aim of this research was to use PCR as a diagnostic tool to *A platys* and to compare this method with positive and negative results obtained through morulae visualization in dog platelets by blood smear evaluation.

MATERIALS 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%, and supposing a prevalence of 50% in an infinite population.[19] Platelet inclusion bodies resembling *A platys*[2] or not were identified in 101 EDTA-blood samples during complete inspection of Diff-Quik (Instantprov®) stained blood smears from dogs, males and females of different breeds and ages, from different localities of the city of Rio de Janeiro. The inclusions were classified as unspecific, meaning negative to *A platys*, or specific, meaning positive to *A platys* (Figures 1 and 2).

The PCR protocol used in this paper proved to be a sensitive and specific method by other authors who had used this protocol for *A platys* diagnosis.[3,20,21] The PCR was

Figure 1. Platelet inclusion bodies in stained Diff-Quik blood smear resembling *A platys* (considered as positive to *A platys* in morphological evaluation) in dogs from Rio de Janeiro, Brazil. Light microscopy (magnification 1000×).

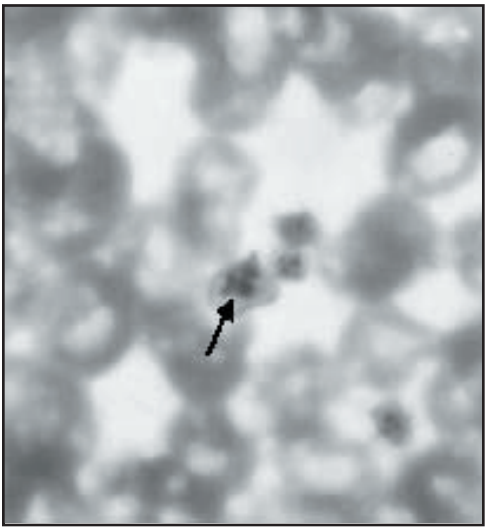
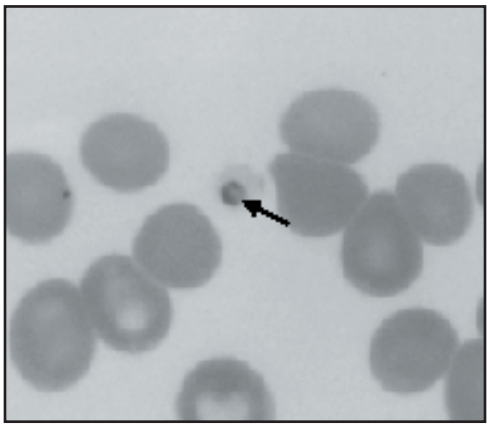


Figure 2. Unspecific platelet inclusion body in stained Diff-Quik blood smear (considered as negative to *A platys* in morphological evaluation) in dogs from Rio de Janeiro, Brazil. Light microscopy (magnification 1000×).



carried out in 2 stages. The first stage 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 *A platys*. The negative samples were not tested in this second reaction. The McNemar test was used to verify the coherence between the blood smear findings and PCR. This non-parametric test was used to determine whether the proportion of blood smear positives and negatives and PCR positives and negatives were equal for both members. It considered PCR as a golden test, comparing correct versus incorrect identification of parasites in a same sample. The data were analyzed using SPSS software v.10.0 (SPSS Inc, Chicago, Illinois, USA).

PCR

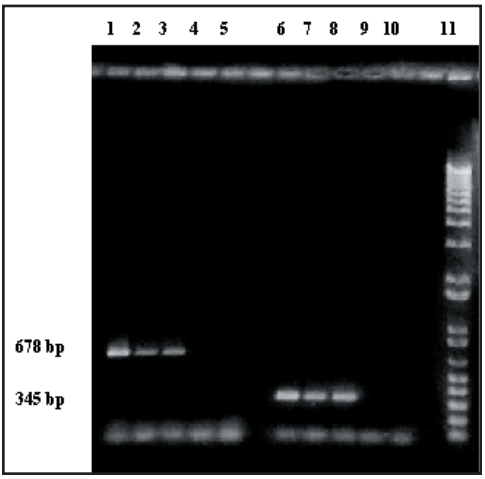
Three controls were used in this research aiming to access PCR efficiency and possible contamination. The positive control consisted of a DNA solution extracted from a blood sample from a dog known to be infected by *A platys*. This positive control was first tested in Universidade Estadual Paulista–Jaboticabal for the species *E canis* proving negative, tested for genus *Anaplasma* being positive, to *A phagocytophilum* being negative, and finally showed positive to *A platys*. To confirm the PCR positivity to *A platys*, the products were used for DNA sequencing.[22] The negative control consisted of a DNA solution from *Escherichia coli* strain K12 DH5α[23] extracted by the boiling method. The control with no DNA consisted of a mixture of reagents without the final adding of DNA (Figure 3).

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*, *Anaplasma phagocytophilum*, *A platys*, *Anaplasma marginale*, *Anaplasma centrale*, *Wolbachia pipientis*, *Neorickettsia sennetsu*, *Neorickettsia risticii*, and *Neorickettsia helminthoeca*.[3,21] This analysis (PCR1) used the forward primer

Figure 3. Agarose gel electrophoresis showing amplification of *A platys* dog samples from Rio de Janeiro, Brazil. Gel electrophoresis of positive *A platys* control to some species of Anaplasmataceae family-PCR1 (8) and *A platys*-PCR2 (3); *A platys*-negative control to some species of Anaplasmataceae family-PCR1 (9) and to *A platys*-PCR2 (4); (5) and (10): controls without DNA; (6) and (7): positive animals in PCR1; (1) and (2): *A platys* PCR2 positives, confirming the positive results for some species of Anaplasmataceae family (PCR1); (11): molecular weight marker (100 bp).



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.[20,21]

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.[20,21,24]

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.[20]

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).

RESULTS

Based on the presence of characteristic inclusions of *A platys* in platelets, 15 (14.85%) of the 101 dogs were positive and 86 (84.16%) were negative. In molecular analysis, 19 (18.81%) were positive in PCR1 and 82 (81.19%) were negative. Testing the positives in PCR1 to *A platys* (PCR2), only 16 (15.84%) had the parasite, increasing the number of negatives to 85 (84.16%).

The results are listed in Table 1. Two animals were negative by PCR1 but positive in morphologic analysis (false-positives) and more 3 positives in PCR1 were negative in PCR2 (false-positive animals). Six dogs were negative in morphologic analysis but positive in PCR1. From those negative animals in morphologic analysis, only 4 were identified as *A platys*. Adding the false-positives and confirming the false-negatives, the conclusion is: 5 false-positive animals (2 in PCR1 and 3 in PCR2) and 4 false-negative animals.

Analyzing the results, the McNemar test revealed, at significance level of 0.05, a coherence between the morphological and molecular findings ($P = 0.289$).

DISCUSSION

Inclusion bodies in platelets can be related to other diseases,[15] as platelets play an important role during inflammation.[25] A common hematological finding during inflammation is platelet activation and release of soluble mediators that control the activity of blood and endothelial cells,[26] thus cytoplasmic inclusion bodies are frequent. These inclusions are characterized by the formation of granules, which concentrate in the center region of the platelet as a false nuclei, resembling a morulae.[27]

Table 1. Results Obtained by Morphological Evaluation of Platelet Inclusion Bodies, PCR1, and PCR2 in Dogs from Rio de Janeiro, Brazil, Presenting at Least 1 Positive Test.

Animals (n = 21)	Inclusion	PCR 1[a]	PCR 2[b]
1	pos	neg	Not done
2	pos	neg	Not done
3	neg	pos	pos
4	neg	pos	pos
5	neg	pos	pos
6	pos	pos	pos
7	neg	pos	neg
8	pos	pos	pos
9	pos	pos	neg
10	pos	pos	pos
11	pos	pos	pos
12	pos	pos	pos
13	pos	pos	pos
14	pos	pos	pos
15	neg	pos	pos
16	pos	pos	pos
17	pos	pos	pos
18	pos	pos	pos
19	pos	pos	pos
20	pos	pos	pos
21	neg	pos	neg
Total	15	19	16

[a]PCR1 for the presence of several species of the family Anaplasmataceae.

[b]PCR2 for the presence of *Anaplasma platys*.

The 2 false-positive samples (animal 1 and animal 2), which were negatives in PCR1 (Table 1), were evaluated separately to be sure of the presence of morulae in the blood smear. In 1 of them, an incorrect diagnosis was considered[10,13,15] and the inclusion could be related to platelet activation.[27] The possibility of an infection by another parasite was not discarded, but was considered doubtful. The suspicion can be explained as the primers used to identify the genus *Anaplasma* (PCR1) would test positive for a number of other species of the family Anaplasmataceae[20,21] and, even so, it rendered a negative result, which could support the possibility of platelet activation.

The second false-positive animal presented with thrombocytopenia and morulae-like inclusion bodies in mononuclear leukocytes and platelets. Although this was strongly suggestive of *E canis* and *A platys* (both belonging to the Anaplasmataceae family), insufficient amplification for this species could be possible if low *A platys* parasitemia was present. The presence of a different organism, such as *Rickettsia rickettsi*, could not be completely discarded either, as it does not belong to the family identified by the primer sequences. As the disease is transmitted by the same vector, it should be considered as a differential diagnosis.[28] However, additional studies are needed to document the presence of such inclusions and serological tests should be included in this investigation.

Looking at Table 1 again, we can see 3 false-positive animals (animals 7, 10, 21). This can be explained as the samples that tested positive to *A platys* during morphological evaluation tested negative to PCR2, which is *A platys*-specific. This result was related to the fact that *Ehrlichia morulae* could also be found in platelets.[14,15] Another hypothesis was the possibility of a low *A platys* parasitemia, which would lead to insufficient amplification for this species[29-32] even though these samples had a positive result in PCR1 that suggests the presence of another parasite in the same family.

From the 86 morphologic negative animals, 6 tested positive in PCR1 (animals 3, 4, 5, 7, 15, 21; table 1), which means they had unspecific inclusions to *A platys* but they are probably infected by Anaplasmataceae members. Referring to the data of PCR2, there were only 4 false-negative animals (animals 3, 4, 5, 15). These dogs did not have characteristic inclusions but were positive by PCR to *A platys*. Therefore, from the 6 morphological negative animals that were positive in PCR1, only 4 tested positive in PCR2, and the other 2 are other members of the Anaplasmataceae family.

The *A platys*-positive results were related to the possibility of this parasite not being found in blood smears during chronic disease stages and in cyclic thrombocytopenia,[9,10,11,17] discarding analysis error.

Immunomediated vasculitis is present during *E canis* infection,[17,28] which can explain the presence of unspecific inclusions in both animals positive to PCR1 and negative to PCR2. Further molecular tests should be performed to confirm this hypothesis. The unspecific inclusions can be a result of the inflammation caused by this agent that can happen together with morulae visualization.

After molecular evaluation of 101 samples, 19 (18.81%) of them tested positive for the family Anaplasmataceae (PCR1); from this group, 16 (15.84%) were positive to *A platys* (PCR2), and the other 3 (2.98%) tested negative to this parasite.

In 2 PCR2-negative dogs (animals 7, 21), the result was likely due to morphologic evaluation, which considered the inclusion not to be characteristic for infection. This could be explained by platelet activation from inflammation[28] caused by another infectious agent belonging to the family Anaplasmataceae, identified by PCR1. Animal 9 (PCR2-negative), however, had typical morulae inclusions and the possibility of evaluation error was discarded. This conclusion was justified by a possible low A platys parasitemia, therefore handicapping target DNA amplification in PCR2.[29-32] Concerning morphological analysis, this animal

belonged to the 3 false-positive *A platys* group (PCR2).

Because the McNemar test revealed coherence between the morphologic and molecular findings, it is possible to conclude that there was no significant difference between tests.

CONCLUSION

This paper shows the need to differentiate unspecific granules from parasitic inclusions, in order to avoid misdiagnosis and unnecessary treatment. An accurate light microscopy analysis seems to be a trustable method to diagnose *A platys* infection. The use of PCR as a diagnostic method is also viable, as it offers more specific resources to identify this parasite.

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