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Molecular characterization of *Anaplasma platys* in dogs in Pelotas city, Southern Brazil

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Abstract: *Anaplasma platys* belong to the Anaplasmataceae family, infecting platelets of dogs, leading to a disease known as canine cyclic thrombocytopenia (CCT) and are transmitted by ixodid ticks (Rhipicephalus sanguineus). The present study aims to assess the occurrence of this parasite in Pelotas, southern region of Rio Grande do Sul State, Brazil, investigate de clinical and hematological abnormalities and perform its molecular detection. Blood samples from 89 dogs were drawn (from jugular or cephalic veins) in order to carry out red blood count (RBC), platelet count and polymerase chain reaction (PCR/nested PCR) followed by product sequencing. The presence of morulae within platelets was investigated. Results obtained with blood smear showed 1.1% for Anaplasma spp. A total of 7/89 samples (7.9%) were positive for *A. platys* by nested PCR. In conclusion, the clinical signs and hematological changes detected were nonspecific and common to others diseases. Therefore, it is imperative the use of specific diagnostic techniques in order to identify the etiologic agents.
Keywords: canine anaplasmosis, Anaplasmataceae, 16SRNA gene, *Anaplasma platys*, canine thrombocytopenia, molecular detection, PCR

INTRODUCTION

Hemoparasitosis are endemic diseases with great importance to public health due to its high prevalence worldwide and geographical spread, usually determined by climate change as well as by access to different ecological niches [1]. These diseases are caused by protozoa or obligate intracellular bacteria that infect different blood cells of vertebrate hosts [2]. These agents are transmitted by blood-sucking ectoparasites [3], may causing anemia, leukopenia and/or thrombocytopenia in the definitive host [4, 5].

Anaplasma platys belongs to the Anaplasmataceae family [6], infecting platelets of dogs, leading to a disease known as thrombocytic canine anaplasmosis or canine cyclic thrombocytopenia (CCT). The disease is characterized by a sudden decrease in platelet counts right after infection, with subsequent decrease of *A. platys* in blood circulation followed by the recomposition of normal platelet values. However, cyclically, at intervals of one to two weeks, the recurrence of parasitemia and thrombocytopenia usually is a common finding. In the chronic phase, there is a tendency for reducing the number of infected platelets and of thrombocytopenia [3, 7, 8]. There are reports of the disease in dogs in the United States [6], France [9], Spain [10], Turkey [11], Tunisia [12], Chile [13], Croacia [14], and French Guiana [15]. *A. platys* have been described in several regions of Brazil, with low prevalence in the extreme south of the country [5] and high prevalence in the central-western [16]. There is evidence that *A. platys* are transmitted through the bite of ixodid ticks. Engorged ticks were examined in Japan and the DNA of *A. platys* were amplified [17].

The Laboratorial diagnosis of these infections has been routinely performed through direct identification of structures compatible with morulae of *A. platys* in platelets from blood samples [18]. However, when the hosts are under chronic or subclinical phase of these diseases, these inclusions are not detected [19]. Serological methods as indirect Immunofluorescence assay (IFA) or enzyme-linked immunosorbent assay (ELISA) and molecular techniques as Polymerase Chain Reaction (PCR) are more sensitive and specific diagnostic methods [20].

Whereas ixodid ticks (Rhipicephalus sanguineus) in dogs are often encountered in the study area, and which are described as responsible for transmission of this hemoparasitosis; we intended to determine the occurrence and molecular detection of *A. platys* in dogs treated at a Veterinary Hospital in Pelotas's city, southern Brazil. Also, we also performed sequence alignment to indicate the identity of the parasite species found.

MATERIAL AND METHODS

This is a descriptive study carried out in the city of Pelotas (31º46'19''S and 52º20'33''W), state of Rio Grande do Sul, Southern Brazil. It was conducted for a period of eighteen months (September 2013 to February 2015) into the Clinics Veterinary Hospital of the Universidade Federal de Pelotas (CVH/UFPel), which provides veterinary care for animals in the region where it operates. Every dog with clinical suspect of hemoparasitosis during the study period, underwent to a routine of clinical and laboratory examinations. The inclusion criteria were the presence or history of tick exposure. Blood samples from 89 dogs were drawn (from jugular or cephalic veins) in order to carry out red blood count (RBC), platelet count and polymerase chain reaction (PCR/nested PCR) followed by product sequencing. The presence of morulae within platelets was investigated through smears of blood and buffy coats. The buffy coat was obtained from 4.5 mL of EDTA whole blood sample transferred to a sterile micro tube and centrifuged at 2,500 g for 10 minutes. Then, the buffy coat was collected to make the smears [21]. After staining with Panotico [22] the slides were visualized by optic microscopic at 1.000 x. EDTA blood samples were then stored and frozen at -20ºC until serological and molecular analyses.

The extraction of DNA from whole blood was performed with the QIAamp DNA Blood Mini Kit (Qiagen®, Valencia, California, and United States) according to the manufacturer's recommendations. The primers (table 1) and thermal sequences used were based on the amplification of a 16S rRNA. The reaction was performed using 1.25U of Taq DNA Polymerase (Invitrogen®), PCR Buffer (10 X PCR buffer - 100nM Tris-HCl, pH 9.0, 500 mM KCl), deoxynucleotide (dATP, dTTP, dCTP and dGTP) (Invitrogen®), 1.5 mM of magnesium chloride (Invitrogen®), 0.5 mM of each primer (Invitrogen®) and sterilized ultrapure water (Invitrogen®). The nested PCR reactions were performed using 1µL of the amplified product (in the first PCR reaction). The amplified products were subjected to horizontal electrophoresis on 1.5% agarose gel stained with ethidium bromide (Invitrogen®) (0.5 µL/mL) in 1X TEB buffer, pH 8.0 (44.58 M Tris-base, boric acid 0.44 M, 12.49 mM EDTA). Results were visualized and analyzed using a UV light transilluminator (2020E) coupled to a computer program for analyzing images (Eagle-Eye II-Stratagene®). Sequencing of the amplified products was carried out using the sequencer ABI PRISM 3700 DNA Analyzer (Applied Biosystems). The BLAST program was used to analyze the nucleotide sequences (BLASTn), in order to search and compare similar sequences into the international database (GenBank) [23] with those sequences obtained.

Table-1. Oligonucleotide primers used for the PCR reactions; sizes of the amplimers and references used for molecular detection of Anaplasma spp.

Agent	Sequence of oligonucleotide	Amplimer size (bp)	Reference
Anaplasma spp. -gE3a -gE10R	5'-CACATGCAAGTCGAACGGATTATTC -3' 5'-TTCCGTTAAGAAGGATCTAATCTCC -3'	932	Massung et al., [24]
Nested Anaplasma sp. -gE2 -gE9f	5'- GGCAGTATTAAAAGCAGCTCCAGG -3' 5'- AACGGATTATTCTTTATAGCTTGCT -3'	546	Massung et al., [24]
Anaplasma platys -Platys F -Platys R	5´- AAGTGCAACGGATTTTTGTC -3´ 5´- CTTTAACTTACCGAACC -3'	504	Inokuma et al., [25]

This study was approved by the Ethics Committee for Animal Experimentation (EAEC) of the Federal University of Pelotas, under permit no. EAEC 2058 (Process no. 23110.002058/2011-15). The dogs were enrolled in the study only after the owners gave their written consent.

RESULTS

It was evaluated 89 dogs, 51 male and 38 female; 40 were older than 48 months; 73 were crossbred and 7, 77 and 5 were domiciled, semi-domicilied and stray dogs, respectively. It was observed that 84.3% (75/89) of the dogs were, on the moment of clinical evaluation, parasitized by Rhipicephalus sanguineus. From those, 80.9% (72/89) were not using drugs for ectoparasites.

Among all dogs studied, the most frequently clinical signs observed were apathy (73/89), pale mucosa (59/89), weight loss (46/89), fever (41/89), lymphadenopathy (33/89) and dehydration (30/89). Other symptoms such as bleeding petechiae, ear bleeding, epistaxis and jaundice were detected in less than 15% of the dogs. Among the dogs n-PCR positive

for *A. platys* the most common clinical signs observed were pale mucosa (7/7), apathy (6/7) and weight loss (3/7). There were no symptoms statistically frequent among the dogs infected with *A. platys* when compared with dogs without this infection. The hematological analysis showed anemia (73/89), thrombocytopenia (46/89), leukopenia (4/89) and leukocytosis (33/89). Among the anemic dogs, 42% were also thrombocytopenic. Normocytic and normochromic anemia was the predominant type (52/89), followed by normocytic and hypochromic (11/89) and microcytic and normochromic (12/89) types. All dogs n-PCR positive for *A. platys* showed anemia and thrombocytopenia.

It was observed morulae characteristics of Anaplasma spp. In platelets of only 1dog (1.1%) in buffy coat stained with the rapid panoptic. The results of PCR showed that, from the 89 samples analyzed 7.9% (7/89) were positive with a 546 bp product amplified by nested-PCR for Anaplasma spp (Figure 1). The amplimers obtained for Anaplasma spp showed 99-100% of identity with *A. platys* sequences previously published in GenBank and found in dogs from Croatia (JQ396431).

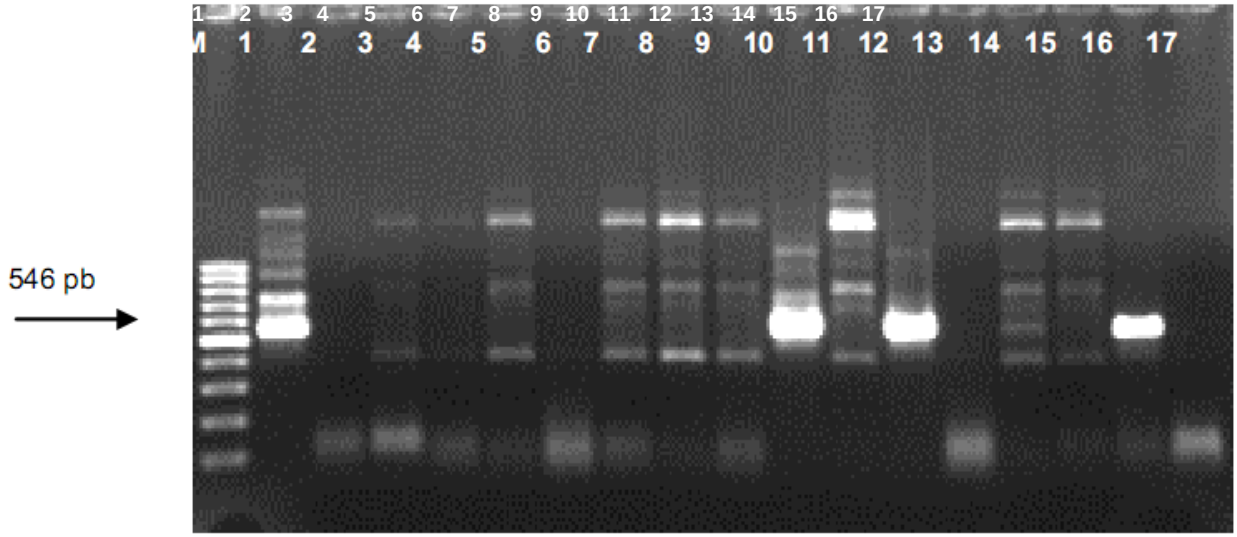

Fig-1: Electrophoresis in agarose gel (1.5%) stained with ethidium bromide. The amplimers shown in the photo are representative of nPCR to Anaplasma spp, obtained using primers gE2 e gE9f. M: molecular weight marker in scale of 100 bp (Invitrogen®); 1: positive control; 2 to 16: blood samples from dogs; 17: White.

DISCUSSION

In order to detect the occurrence of *Anaplasma platys* in the Pelotas region, all dogs presented during the experimental period of suspects hemoparasitosis and history of exposure to ticks were subjected to evaluation of clinical and hematological parameters. By analyzing the most prevalent clinical signs in n-PCR positive dogs for *A. platys*, we found that over 80% had pale mucous membranes and apathy. However, the same symptoms were equally prevalent in non-infected dogs. The clinical signs were nonspecific and without association to the etiological agent focused on this study. Likewise, the most prevalent hematologic changes (normocytic and normochromic anemia and thrombocytopenia) were a common manifestation in *A. platys* infection and others hemoparasitosis as *Babesia canis* and *Ehrlichia canis* [3, 26], which reinforces the necessity for an association between clinical findings and more accurate clinical laboratory methods, in order to identify these agents. In this sense, the clinical signs and hematological changes observed in our study agreed with those described previously [3, 5, 27].

Anaplasma platys are tick-borne bacteria that cause severe disease in dogs [28]. The agent is transmitted by ticks, mainly by Rhipicephalus sanguineus [29]. In Brazil, Rhipicephalus sanguineus is also the most common ixodid, with a prevalence of 6.7% of infected dogs in rural areas and ranging from 56.8% to 93.2% in dogs not domiciled in urban and peri-urban areas [30, 31]. Over the years, the indiscriminate use of acaricides has caused resistance to these drugs which has been the focus of major concern worldwide [32]. In our study, 84,3% of the dogs were parasitized by R. sanguineus at the moment of the clinical evaluation. Our results are in agreement with the literature on the tick transmission of this hemoparasitosis [26, 30, 31, 33].

It was observed a low prevalence of morulae on smears of blood buffy coat samples, which is consistent with the findings described previously [7, 27]. This test is easy to perform and with low cost, but it has low sensitivity and high specificity, allowing the detection of inclusions of different hemoparasites [34, 35]. However, only detects inclusions in the acute phase of the disease. The absence of morulae on direct

examination is not indicative of the absence of agent in the animal organism. In our study we found morulae characteristics of *Anaplasma platys* in only 1 dog (1.1%) while PCR detected the agent in 7 canines (7.9%) of the experiment. The low sensitivity of the method underestimates the true prevalence of the infection and can mask the final diagnosis.

It is worth remembering that PCR is currently considered as the gold standard for diagnosis and detection of specific DNA sequences of the pathogens of these hemoparasitosis, providing the detection of these agents in all the infection stages. Thus, it can be used as a tool monitoring the treatment; in the identification of the infecting species, even when in low concentrations in the blood [7, 36]. Comparing the n-PCR results with results of other studies [5, 16], we consider that the prevalence of infection with *A. platys* in our region is lower than in other areas of Brazil. Lasta *et al.;* (2013) conducted a similar study in Porto Alegre, a city about 300 km from our region and detected a prevalence of 14.07% of the agent in the samples. We believe that this is due to differences in the vector competence of the R. sanguineus [37].

The main objective of this study was confirmed by the presence of *A. platys* in the dogs with suspect of hemoparasitosis, as well as the species and subspecies involved in the infections of dogs from the city of Pelotas. The amplimers obtained for Anaplasma spp showed 99-100% of identity with *A. platys* sequences previously published in GenBank and found in dogs from Croatia (JQ396431). Such a finding is in agreement with the literature reported in other regions of Brazil and the world, showing a close molecular similarity, which demonstrates low variability among geographic regions [5, 36, 38].

CONCLUSION

Molecular methods confirmed the presence of *A. platys*, the etiologic agent of thrombocytic canine anaplasmosis in dogs from the region covered by the Veterinary Hospital, city of Pelotas, southern of Rio Grande Sul State (Brazil). The genera and species identified showed genetic similarity to those diagnosed in the other regions of Brazil. Among the diagnostic methods used, the evaluation of smears of blood buffy coat showed low sensitivity and high specificity when compared to the molecular method. Finally, the clinical signs and haematological changes detected were nonspecific and common to others diseases. Therefore, it is imperative the use of specific diagnostic techniques in order to identify the etiologic agents. This is the first molecular detection of these agents in this area.

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