Scholars Journal of Agriculture and Veterinary Sciences **e-ISSN 2348–1854**
Sch J Agric Vet Sci 2015; 2(5):337-341 **p-ISSN 2348–8883**

(An International Publisher for Academic and Scientific Resources) DOI: 10.36347/sjavs.2015.v02i05.001

Parasitologic, Serological and Molecular Diagnostic of *Ehrlichia canis* and *Babesia canis* in a Veterinary Hospital in Southern Brazil

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Abstract: *Ehrlichia canis* and *Babesia canis* are etiologic agents of canine hemoparasitosis, which normally share a common vector; ixodid ticks (Rhipicephalus sanguineus). This study aims to detect these agents in 89 blood samples from dogs treated at a Veterinary Hospital in Pelotas's city, southern Brazil, through parasitological, serological and molecular methods. The presence of compatible structures was investigated (morulae within leukocytes and piroplasms in erythrocytes) by means of blood and buffy coat smears. Immunoglobulins IgG anti-*E. canis*and *B. canis* were detected in 21.4% and 22.5% of the samples respectively. PCR for *E. canis*and *B. canis* were positive in 37.1% and 10.1% of samples. After sequencing, genera and species were identified genetic similarity with those diagnosed in the other regions of the country. This paper reports the first molecular detection of *E. canis*and *B. canis* in dogs' blood samples in the city of Pelotas - RS, southern of Brazil.
Keywords: ehrlichiosis; canine babesiosis; PCR; *Babesia canis* vogeli; hemoparasites; canine blood parasites

INTRODUCTION

Tick-borne hemoparasitic diseases are endemic worldwide and of great significance to public health due to their high prevalence and geographical distribution. In Brazil, the hemoparasites most commonly transmitted to dogs by ixodid ticks are *Ehrlichia canis* [1] and *Babesia canis* [2]. *Ehrlichia canis* is an intracellular parasite, especially of monocytes and lymphocytes, and is considered the most important species in Brazil, causing the disease known as Canine Monocytic Ehrlichiosis (CME)[3]. The acute phase of CME is characterized by anemia, leukopenia and thrombocytopenia, while the subclinical phase is usually asymptomatic, with elevated antibody titers and mild hematologic changes [3, 4].

Babesia canis infects red blood cells (RBC) and is the causative agent of the disease known as Canine Babesiosis [5]. Common manifestations of the disease are anorexia, pallor, weight loss, fever, tachycardia, splenomegaly, jaundice, and hemoglobinuria, normally resulting from hemolytic anemia and thrombocytopenia [6, 7].

The main hemoparasites in Brazil are routinely diagnosed in the laboratory through the direct identification of structures compatible with *E. canis*morulae in leukocytes [8, 9] and of *B. canis* piroplasms in erythrocytes of blood samples [10, 11]. However, when the host is in the chronic or subclinical phase of these diseases, the aforementioned inclusions are not visible, hindering the etiologic diagnosis of these diseases and hence, the correct choice of treatment [12]. Other diagnostic methods that are more sensitive and specific, such as the isolation of agents in cell cultures, indirect immuno fluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), western blotting, and polymerase chain reaction (PCR), are well described in the literature [13, 14].

Considering the similarity of clinical signs with other viral and bacterial infections, neoplastic and immune-mediated diseases, there is urgent need for a more accurate diagnosis of these hemoparasites. Therefore, the focus of this study was on the parasitological, serological and molecular detection of *E. canis* and *B. canis* in dog blood samples suspected of these both agents treated at the Clinical Veterinary Hospital of the Federal University of Pelotas (CVH/UFPel). We also performed sequence alignment to indicate the identity of the parasite species found.

MATERIAL AND METHODS

This is a descriptive study conducted for a period of one year at the Veterinary Hospital of the Federal University of Pelotas (CVH/UFPel), which provides veterinary care for animals in the surrounding region. Every dog with clinical suspicion of hemoparasites during the period of this study underwent routine clinical and laboratory exams. The dog owners answered a questionnaire about their animal's normal habits, area of residence (urban or rural), access to streets, age, and history of tick infestation. The ectoparasites were collected and examined under a stereoscopic microscope. The identification of ticks was based on dichotomous keys as proposed in literature [15, 16]. The inclusion criteria were the presence or history of tick exposure.

Blood samples of 89 dogs were collected by jugular or cephalic venipuncture. The presence of compatible structures was investigated, e.g., morulae within leukocytes and piroplasms in erythrocytes, by means of blood and buffy coat smear. 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 [13]. After staining with Panotico [17] 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 presence of anti-E.canis and *B. canis* IgG antibodies in the sera was detected by Indirect Immuno fluorescence Assay (IFA). *E. canis*antigen was obtained from DH82 culture cells infected with *E. canis*(Jaboticabal strain) at the Immuno Parasitology Laboratory, UNESP [18]. The B. vogeli antigen was prepared by inoculation intravenously into a splenectomized three-month-old dog negative for hemoparasites by PCR and serology. Blood smears were performed twice a day to check for the presence of parasites in microscopic examination of Giemsa-stained. The parasitaemia peak occurred on the fifth day after inoculation; infected blood was collected with Alsever solution (113.7 mM glucose, 27.2 mM sodium citrate, 71.8 mM sodium chloride) (LabSinth, Diadema, São Paulo, Brazil). The slides containing air-dried fixed B.canis vogeli trophozoite-infected blood were used in IFA [14]. Prior to its use, IFA slides were stored frozen at -20ºC. Antigen slides were removed from storage and allowed to thaw at room temperature during 30 minutes. Ten microliters of twofold dilutions of sera (cut-off of 1:20 for *E. canis*and 1:40 for *B. canis*) were placed in wells on antigen slides. Known positive sera were obtained from symptomatic dogs with ehrlichiosis and babesiosis. Negative control sera were obtained from dogs that had not been exposed to the agents, according to negative PCR and IFA tests. Slides were incubated at 37ºC in a moist chamber for 45 min, washed 3 times in PBS (pH 7.2) for 5 min, and air dried at room temperature. FITC-labeled anti-dog conjugate (Sigma-Aldrich, St. Louis, MO, USA) was diluted according to the manufacturer and added to each well. These were incubated again at 37ºC, washed 3 times in PBS, once more in distilled water and air dried at room temperature. Slides were overlaid with buffered glycerin (pH 8.7) and examined with a fluorescence microscope.

DNA was extracted from 200 µL of whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN®, Valencia, California, USA), following the manufacturer's instructions. The primers and thermal sequences used for each agent were based on the amplification of the 16S rRNA portion of E.canis [19] and the 18S rRNA portion of *B. canis* gene [20]. The reaction was performed using 1.25U of DNA Taq 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 positive control DNA for *E. canis*was obtained from DH82 cells infected with the Jaboticabal strain [18], and *B. canis* vogeli infected blood sample was obtained from a dog experimentally infected with Jaboticabal strain [14]. Ultra-pure sterile water was used as negative control. 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). The results were visualized and analyzed using a UV light transilluminator (2020E) coupled to animating system (*Stratagene Eagle Eye II*). Sequencing of the amplified products was carried out using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems). The BLAST program was used to analyze the nucleotide sequences (BLASTn), in order to search for and compare sequences in the international database (GenBank) [21] similar to those obtained in this study.

RESULTS

The animals that participated in this study were 89 dogs, 51 males and 38 females; 40 were older than 48 months; 73 were crossbreeds, and 7, 77 and 5 were domiciled, semi-domiciled and stray dogs, respectively. During the clinical evaluation, 84.3% of the dogs were found to be parasitized by Rhipicephalus sanguineus, and 80.9% of the parasitized animals were not on anti ecto parasitic drugs therapy.

Piroplasms characteristic of Babesia spp. were observed in erythrocytes in 2.3% of the blood smears and, morulae characteristic of Ehrlichia spp. in mononuclear leukocytes in 2.3% of samples of the buffy coat smears stained by rapid panotic staining.

Of the 89 sera samples analyzed by Indirect Immuno fluorescence Assay (IFA), 21.4% were positive for E. canis, and 22.5% for *B. canis*. The antibody titers ranged from 1:20 (cut-off) to 1:2560 (E. canis); and from 1:40 (cut-off) to 1:640 (*B. canis*). Three samples tested seropositive for both antigens.

The PCR results showed that, of the 89 analyzed samples, 37.1% were positive with a 358 bp product amplified by nested PCR (nPCR) for *E. canis*and10.1% for *B. canis* (PCR amplimers with 400 bp– with restriction pattern consistent with *Babesia canis* vogeli). Three dogs seropositive for both antigens were PCR positive too. The amplimers obtained for *E. canis*showed 98-99% identity to each other, and a high degree of identity with the sequences published in GenBank. A single DNA sample showed identity to *E. canis*sequences in dogs from Mexico (EF424612) and Brazil (EF195134). *B. canis* DNA showed 99% similarity to *B. canis* vogeli isolates from dogs in Brazil (AY371198), Italy (AY072925) and France (JX304677).

DISCUSSION

*E. canis*and *B. canis* are parasites that infect blood cells, leading to the manifestation of severe diseases in domestic dogs. The aim of this study was to detect the presence of these hemoparasites in dogs with clinical signs suggestive of hemoparasitosis, based on parasitological, serological and molecular assays. The transmission of these diseases usually occurs mechanically, with Rhipicephalus sanguineus described as the main vector in canines. In the case of Babesia spp. there is biological multiplication inside ticks [22]. Among the dogs in this study, 37.1% and 10.1% were PCR positive for *E. canis*and *B. canis* respectively. Additionally, 80.9% of the dogs were parasitized by R. sanguineus at the time they were clinically evaluated. Thus, our findings regarding the tick-borne transmission of these hemoparasites are in agreement with those previously reported [23, 24]. Also, the occurrence of co-infection (positive results in PCR and IFA) in three samples, suggests the transmition by the same tick for both agents.

It was observed a low frequency of morulae and piroplasms in samples of blood and buffy coat smears, which are consistent with the findings, described by others researchers [25, 26]. These tests are inexpensive and easy to perform, but it has low sensitivity and high specificity, allowing for the detection of inclusions of different hemoparasites [9, 10]. Furthermore, these inclusions are characteristic of the acute stage of the infection and do not occur in sub clinic and chronic phases.

Until the present moment, most diagnoses of hemoparasitosis in the study area were based on clinical history, symptoms and detection of inclusions in blood smears. This is the first study of detection of these agents by serological and molecular methods. By serological technique we obtain 21.4% positive for E. canis. However, the PCR detected 37.1% positive for this agent. Some authors refers PCR as a highly sensitive and specific method, and its sensitivity is low when blood samples are collected from naturally asymptomatic infected dog in the chronic phase of the disease [22]. PCR was considered the gold standard for the diagnosis and detection of specific DNA sequences of the pathogens of these hemoparasites, enabling the detection of these agents in all the stages of infection, even before seroconversion [10]. Thus, PCR can be used as a tool for monitoring treatment, and for the identification of infecting species, even when present in low concentrations in the blood [3, 11, 27]. In contrast, serological tests detect antibodies, on average, on the 21stday post-infection, and titers usually remain elevated during the subclinical and chronic phases; exams performed during the first days post-infection (prepatent period) may result in false-negative responses. The clinical sample of this study included symptomatic animals, which were possibly in the acute phase of the disease. This probably explains the low frequency of serological tests for E. canis, since these animals may not have had enough time to produce IgG antibodies.

Despite the high IFA specificity, a relatively large number of false-positive results were observed for *B. canis* when compared with PCR. To *B. canis* by serological technique we obtain 22.5% positive cases, and by PCR 10.1%. In our study, the presence of seropositive dogs to *B. canis* showing negative results in PCR suggests the occurrence of sub clinic or chronic phase of the disease; or these dogs were previously exposed to the agent, maintaining detectable antibody levels. Also, this identification (antibodies to *B. canis*) suggests the possibility of a cross-reaction, or that the animals were taken for clinical evaluation due to other diseases with similar symptoms, revealing exposure titers to the parasite during the laboratory evaluation.

The main objective of this study was achieved through the confirmation of the presence of *E. canis*and *B. canis* (vogeli) in dogs with suspicion of hemoparasitosis, as well as the species and subspecies involved in the infections of dogs from the city of Pelotas. This finding is in agreement with reports in the literature from other regions in Brazil and around the world, showing close molecular similarity and thus demonstrating low variability among geographic regions [28, 29].

CONCLUSIONS

The presence of *E. canis*and *B. canis* (vogeli), the etiologic agents of hemoparasitosis, was confirmed by the molecular methods employed in this study. This identification was carried out in dogs from the region surrounding the Veterinary Hospital in the city of Pelotas, in the southern part of the state of Rio Grande do Sul (Brazil).The identified genera and species showed genetic similarity to those diagnosed in other regions of Brazil. This is the first molecular detection of these agents in this area. Future studies involving other Anaplasmataceae agents in small and wild animals are needed.

ACKNOWLEDGMENTS

We are grateful to Dr. Rosangela Zacarias Machado by kindly supplied the antigen used.

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