Available online at www.sciencedirect.com
ScienceDirect
The Veterinary Journal 179 (2009) 145–148
The Veterinary Journal
www.elsevier.com/locate/tvjl

Short Communication

Molecular evaluation of the incidence of *Ehrlichia canis*, *Anaplasma platys* and *Babesia* spp. in dogs from Ribeirão Preto, Brazil

Flávia Santos [a], Juliana S. Coppede [a], André L.A. Pereira [a], Letícia P. Oliveira [a], Patrícia G. Roberto [a], Roberta B.R. Benedetti [c], Lenise B. Zucoloto [c], Flávia Lucas [d], Lúcia Sobreira [d], Mozart Marins [a,b,*]

[a] *Unidade de Biotecnologia, Universidade de Ribeirão Preto (UNAERP), Ribeirão Preto, SP, Brazil*
[b] *Heranza – Biotecnologia, Ribeirão Preto, SP, Brazil*
[c] *Curso de Medicina Veterinária, Centro Universitário Barão de Mauá, Ribeirão Preto, SP, Brazil*
[d] *Curso de Medicina Veterinária, Centro Universitário Moura Lacerda, Ribeirão Preto, SP, Brazil*

Accepted 17 August 2007

Abstract

Canine monocytic ehrlichiosis caused by *Ehrlichia canis* is endemic in many regions of Brazil. Since thrombocytopenia is a common finding in infected dogs, many clinicians tend to use it as an indication for antibiotic treatment. Polymerase chain reaction (PCR) and nested PCR were used to study the presence of *E. canis*, *Anaplasma platys* and *Babesia* spp. in thrombocytopenic and non-thrombocytopenic dogs from Ribeirão Preto, Brazil. Despite the high prevalence of *E. canis* infection among thrombocytopenic dogs, 46.7% of the thrombocytopenic dogs studied were either infected with *Babesia* spp. or *A. platys* or not infected with any of the three pathogens. There was a high incidence (25.4%) of *E. canis* infection in non-thrombocytopenic dogs. Although infection with *E. canis* should be considered in thrombocytopenic dogs, the final diagnosis needs to be confirmed by complementary tests such as blood smears and PCR to avoid the unnecessary use of antibiotics.

Keywords: *Ehrlichia canis*; *Anaplasma platys*; *Babesia* spp.; Nested PCR; Canine monocytic ehrlichiosis

In Brazil, the first case of canine monocytic ehrlichiosis (CME) was reported in 1973 in a dog from Belo Horizonte, Minas Gerais (Costa, 1973). Today, the disease is found throughout the country and is considered to be endemic in many regions. After routine clinical examination of suspected animals, and in the absence of any characteristic clinical sign of other diseases, most veterinarians tend to rely on thrombocytopenia to diagnose CME, followed by antibiotic treatment with no identification of other possible causative agents. Although a common finding in CME-infected dogs, thrombocytopenia can also be a manifestation of other diseases, as well as in infections with other *Ehrlichia* spp. and parasites (Harrus et al., 1999).

The area around São Paulo State is considered to be endemic for *Ehrlichia canis*. In a study conducted in the town of Botucatu, located 240 km from Ribeirão Preto, nested polymerase chain reaction (PCR) was used to detect *E. canis* infection in thrombocytopenic and non-thrombocytopenic dogs (Bulla et al., 2004). Among dogs with a platelet count <100,000, 63% were found to be infected and the authors suggested that animals with severe thrombocytopenia should be screened for ehrlichiosis. However, most veterinarians apply the reference parameter of <200,000 as an indication of thrombocytopenia. Using this value as a reference, the number of infected animals falls to 45.2% in that study.

* Corresponding author. Address: Unidade de Biotecnologia, Universidade de Ribeirão Preto (UNAERP), Ribeirão Preto, SP, Brazil. Tel.: +55 16 3603 6892; fax: +55 16 3603 7030.
E-mail address: marins@heranza.com.br (M. Marins).

1090-0233/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tvjl.2007.08.017

In a similar investigation in Rio de Janeiro, located 712 km from Ribeirão Preto, Macieira et al. (2005) confirmed the higher prevalence of *E. canis* in thrombocytopenic dogs when compared to non-thrombocytopenic animals. However, among thrombocytopenic dogs, less than one-third of the animals (26.8%) were infected with *E. canis*. Analysing blood smears, the authors suggested the presence of other parasites such as *Babesia* spp. and *Anaplasma* spp. Therefore, thrombocytopenia and other clinical signs used as an indication for *E. canis* infection can no longer be considered sufficient to establish the diagnosis of CME. Moreover, in view of the high incidence of other parasites in Brazil, the correct identification of the parasite (or of other diseases as the cause of thrombocytopenia, especially when *E. canis* has been ruled out) continues to be a problem for the clinician. In the present study we used PCR and nested PCR to determine the incidence of *E. canis*, *Babesia* spp. and *Anaplasma platys* in dogs from Ribeirão Preto.

The animals were seen at the clinics of two private Veterinary University Hospitals between October and December 2005. EDTA-anticoagulated whole blood was collected from 221 dogs and an aliquot of each blood sample was used for the extraction of genomic DNA using the GFX Genomic Blood DNA Purification Kit (Amersham Biosciences). The remaining sample was used for manual platelet count (Jannini and Jannini-Filho, 1984). The dogs were divided into two groups: thrombocytopenic (<200,000 platelets/μL) and non-thrombocytopenic (≥200,000 platelets/μL).

Nested PCR was used for the detection of *E. canis* and *A. platys* (Bulla et al., 2004; Dawson et al., 1994). All primers were based on those previously described in the literature and sequences deposited in GenBank. Briefly, in the first reaction the newly designed forward primer Aplasense 5′-CTCAGAACGAACGCTGGCGGCAAGC-3′ and the previously described reverse primer ECB 5′-CGTATTACCGCGGCTGCTGGC-3′ (Dawson et al., 1994) were used to amplify the 16S rRNA gene from both species. In the case of *E. canis*, these primers generated a fragment of 481 bp, encompassing position 1–481 of the sequence deposited in GenBank (AF162860), while for *A. platys* a fragment of 476 bp was generated, encompassing position 1–476 of the sequence deposited in GenBank (AF286699).

In the second reaction, 1 μL of the first reaction was used with the species-specific primers designed to amplify specific targets of the 16S rRNA gene: ECA 5′-CAATTATTTATAGCCTCTGGCTATAGGAA-3′ (Wen et al., 1997) and HE-3 5′-TATAGGTACCGTCATTATCTTCCCTAT-3′ (Dawson et al., 1994) for *E. canis*, which generated a 389-bp fragment, encompassing position 49–437 of the GenBank sequence, and the previously described sense primer *E. platys* primer 5′-GATTTTTGTCGTAGCTTGCTA-3′ (Kordick et al., 1999) and the newly designed reverse primer Aplastintrev3 5′-GGTACCGTCATTATCTTCCC-3′ for *A. platys*, which generated a fragment of 382 bp, encompassing position 47–428 of the sequence deposited in GenBank.

A single PCR was performed for the detection of *Babesia* spp. using primers that amplify 18S rRNA gene targets from a wide range of *Babesia* species: the previously described sense primer BabgenF 5′-GAAACTGCGAATGGCTCATTA-3′ (Baneth et al., 2004) and the newly designed reverse primer Babesiarev1 5′-CCATGCTGAAGTATTCAAGAC-3′, which generated a 642-bp fragment, encompassing position 81–722 of the GenBank sequence (AY072926). As a positive control, cytdogFWD 5′-CATCAGTCACCCACATCTGC-3′ and cytdogREW 5′-CCATGAATGCTGTGGCTATG-3′ primers were used for amplification of the mitochondrial cytochrome b gene, which produced a 198-bp fragment. PCR and nested PCR were performed in a PT-100 thermocycler (MJ Research) consisting of an initial step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. After a final step at 72 °C for 10 min, the reaction was stored at 10 °C. A 10-μL aliquot of each reaction was analyzed by agarose gel electrophoresis. The amplified products were purified and sequenced in an automated DNA sequencer (model 377, Applied Biosystems). Fig. 1 illustrates an example of the results obtained.

In contrast to previous investigations (Bulla et al., 2004; Macieira et al., 2005), a marked increase in the incidence of *E. canis* infection was observed among non-thrombocytopenic dogs. We believe that the reason for this conflicting result is the nested-PCR procedure. Most authors use as a substrate in the second reaction, which is species specific, only the positive reactions of the first PCR whose primers detect a wide range of species. In contrast, we screened positive and negative samples of the first reaction in the second amplification. To ensure the absence of contamination, we also used the negative controls of the first reaction in the second reaction and they were always negative. Eighty-six (38.9%) of the 221 animals studied were positive for *E. canis*, with 57 (66.3%) being classified as thrombocytopenic and 29 (33.7%) as non-thrombocytopenic. Moreover, we observed thrombocytopenic dogs that were infected with *A. platys* or *Babesia* spp. either as a single infection or as a co-infection with *E. canis*. *A. platys* and *Babesia* spp. were identified in 33 (14.9%) and 18 (8.1%) of the animals studied, respectively. Twenty-seven (81.8%) *A. platys*- and 17 (94.4%) *Babesia* spp.-positive animals were classified as thrombocytopenic. Among the 221 animals studied, 57/107 (53.3%) of thrombocytopenic animals and 29/114 (25.4%) of non-thrombocytopenic animals were positive for *E. canis* infection. Considering co-infection with *A. platys* or *Babesia* spp. together with *E. canis*, the number of infected animals increased from 57 (53.3%) to 85 (79.4%) among thrombocytopenic animals, corresponding to an increase of 26.1%. However, the increase was only 4.4%, from 29 to 34 (29.8%), in non-thrombocytopenic animals.

Although the incidence of *E. canis* infection was higher among thrombocytopenic dogs than among non-thrombo-