Vet. Res. 35 (2004) 141–146
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DOI: 10.1051/vetres:2003038

Short note

The relationship between the degree of thrombocytopenia and infection with *Ehrlichia canis* in an endemic area

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(Received 14 March 2003; accepted 14 August 2003)

Abstract – *Ehrlichia canis* is the causative agent of canine monocytic ehrlichiosis. In order to evaluate platelet counts as a screening test for *E. canis* in an endemic area, 217 whole blood samples from dogs were divided into three groups: 71 non-thrombocytopenic samples (group A, platelet counts greater than 200 000/µL) and 146 thrombocytopenic samples (less than 200 000/µL). The thrombocytopenic group was further divided into 62 with platelet counts between 100 000–200 000/µL (Group B) and 84 samples with less than 100 000 platelets/µL (Group C). All samples were examined for the presence of a segment of the *Ehrlichia canis* 16S rRNA gene using a nested polymerase chain reaction. Sixty-seven of the 217 samples (30.9%) were positive for the presence of the *E. canis* 16S rRNA gene; 53 (63.1%) of the group C samples and 13 (21%) of group B. Only one (1.4%) of the non-thrombocytopenic samples (Group A) was positive. These data support the concept that platelet counts may be a good screening test for canine monocytic ehrlichiosis, and that the magnitude of thrombocytopenia may increase the reliability of diagnosis.

***Ehrlichia canis* / thrombocytopenia / platelet counts / screening / PCR**

1. INTRODUCTION

Ehrlichia canis is an obligate, intracytoplasmic parasitic disease that affects the canidae and is the causative agent of canine monocytic ehrlichiosis (CME) [6, 18]. The disease is transmitted through the saliva of the brown dog tick, *Rhipicephalus sanguineus*, and has a worldwide distribution [6, 10]. Once an animal is infected, the syndrome progresses through several phases: acute, subclinical and chronic. Each stage can be characterized by an assortment of clinical and hematologic abnormalities [18]. The most prevalent hematological abnormality in all stages of the disease is

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thrombocytopenia, approximately 84% of all cases [13]. The magnitude of the thrombocytopenia can vary between the different stages. In the subclinical phase, the thrombocytopenia may be mild to non-existent while in the acute and chronic phases, it is invariably profound [3, 14]; indeed typically in the chronic phase, there is a concurrent pancytopenia [6]. The mechanisms of the thrombocytopenia may involve immune destruction (mainly in the acute phase) [16], decreased production (chronic phase) [6], increased platelet consumption [6, 18], decreased platelet half-life [11], splenic sequestration [8] or it may be secondary to increased concentrations of circulating platelet migration-inhibition factor [1].

With such a high prevalence of thrombocytopenia in dogs infected with *E. canis*, the use of platelet counts as a screening test for the disease has been proposed [13]. Screening tests are used in a clinical setting for the presumptive identification of a positive specimen that then requires confirmation by a diagnostic test. Diagnostic tests are used to distinguish between animals that have the disease in question from those with other related diseases. With proper quantitative techniques, platelet counts can be an inexpensive and reliable method for screening a population of dogs in an endemic area and direct further diagnostic testing to confirm the disease.

The relationship between the magnitude of thrombocytopenia and the prevalence of the disease has not been established. Accordingly, in order to determine the relationship between the degree of thrombocytopenia and the possible infection with *E. canis* in an endemic area, the presence of *E. canis* was presumed on the basis of a positive nested PCR assay for the presence of the 16S rRNA gene segment. By better defining the relationship between platelet counts and the presence of *E. canis*, greater credibility can be placed on this screening procedure.

Usually before these diagnostic tests are employed, screening tests are used in an attempt to rationalize selection of further diagnostic procedures. A common screening test for an *E. canis* infection is the use of peripheral blood platelet counts [13].

2. MATERIALS AND METHODS

2.1. Thrombocytopenic and non-thrombocytopenic blood samples

Two hundred and seventeen canine whole blood EDTA samples were randomly obtained from routine submissions to the Clinical Pathology Laboratory service of Faculdade de Medicina Veterinaria e Zootecnia of the Universidade Estadual Paulista at Botucatu city, in the countryside of São Paulo State, Brazil. A complete blood count was performed on all samples using an automated hematology analyzer (Abbott Cell Dyn 3500R, Abbott Park, IL, USA), each counting was confirmed by systematic analysis of blood smear stained by a Romanowsky stain, which was scanned in order to check the presence of platelet aggregates. The selected blood samples were divided into 71 non-thrombocytopenic samples (platelet counts greater than 200 000/μL, group A) and 146 thrombocytopenic samples (less than 200 000/μL). The thrombocytopenic group was further divided into samples with platelet counts between 100 000–200 000/μL (n = 62, group B) and those with less than 100 000 platelets/μL (n = 84, group C).

2.2. Nested polymerase chain reaction of whole blood samples for *E. canis*

All blood samples were examined for the presence of the *E. canis* organism using a described nested PCR technique [17]. For the first PCR reaction, genomic DNA was extracted from the whole blood using the GFX Genomic Blood DNA Purification Kit® (Amersham, Biosciences, Piscataway, NJ) following the manufacturer's recommendations.

Approximately 0.5 to 1.0 μg of the genomic DNA was used as a template to

amplify a fragment of the *E. canis* 16S rRNA gene. Each PCR reaction mixture contained 1× reaction buffer (50 mM KCL, 20 mM Tris-HCl (pH 8.4), 0.1% Triton X-100), 1.75 mM $MgCl_2$, 0.2 mM dNTP, 1 μM of primers specific for 16S rRNA of the *Ehrlichia* genus EHO sense and EHO antisense [18], 0.625 U Taq DNA polymerase and autoclaved ultrapure water brought to a final volume of 25 μL. The thermocycle profile included an initial denaturing step at 94 °C for 10 min then 40 cycles of 94 °C denaturing for 60 s, primer annealing at 60 °C for 60 s then primer extension at 72 °C for 60 s. The final step was primer extension at 72 °C for 4 min then held at 4 °C. After completion of the PCR, 10 μL of the reaction product was separated on a 1.5% agarose gel with ethidium bromide added in Tris-Borate EDTA (TBE) at 90 volts for approximately 1 h.

Those PCR reactions that resulted in positive amplification of a segment of the *Erhlichia* sp. 16S rRNA were taken through a second PCR amplification. The second PCR reaction was identical to the first PCR with the exception of the template and primers used. The template for the second nested PCR reaction was a 1.0 μL aliquot of the positive initial reaction. Primers used in the nested PCR were specific for a segment of the *E. canis* 16S rRNA and were designed from a published sequence [17]. The pair of primers used for the initial reaction was the following: EHO sense 5'-AGAACGAACGCTGGCGGCAAGCC-3' and EHO antisense 5'-CGTATTACCGCGGCTGCTGGC-3'. The pair of primers for the nested reaction was the following: ECA sense 5'-CAATTATTTATAGCCTCTGGCTATAGGAA-3' and ECA antisense 5'- TATAGGTACCGTCATTATCTTCCCTAT-3'.

For every PCR batch, ultra-pure autoclaved water was used as a template and acted as a negative control. Also within each PCR run, genomic DNA from a confirmed case of *E. canis* was used as a positive control.

2.3. Statistical analysis

The proportions for positivity for *E. canis* in the three groups (A, B and C) were compared by using the Chi-square test. First, the hypothesis of the homogeneity of the proportions in the three groups was tested (chi-square with 2 df). Since the above hypothesis was rejected, groups B and C were combined and contrasted with group A (chi-square with 1 df), followed by the contrast between groups B and C (chi-square with 1 df).

Statistical significances were stabilized at the level of 5% ($p < 0.05$).

3. RESULTS

A positive amplification of the *Ehrlichia* sp. 16S rRNA in the initial PCR reaction yielded a 478 base pair (bp) product. The use of this product as a template for the nested PCR using the *E. canis* specific primers resulted in a 389 bp product (Fig. 1).

Sixty-seven (30.9%) of the 217 whole blood samples screened for the presence of *E. canis* 16S rRNA using the nested PCR procedure were positive. The animals were further divided into groups; 63.1% (53/84) of group C and 21% (13/62) of group B dogs were positive for the presence of *E. canis* (Fig. 2). Only 1.4% (1/71) of the non-thrombocytopenic dogs (group A) was positive for *E. canis* (Fig. 2). Group A presented a statistical difference from the other two groups ($p < 0.001$) and the groups A and B were different from each other ($p < 0.001$).

4. DISCUSSION

The results of this study suggest that the use of platelet counts as a screening test for the presence of an *E. canis* infection in dogs in an endemic area may be a viable tool for

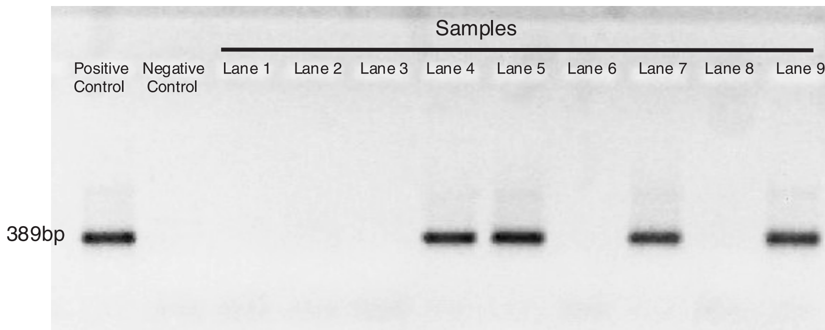

Figure 1. Representative electrophoretic agarose gel results for the nested PCR assay testing for the presence of *E. canis* 16S rRNA gene in thrombocytopenic (groups B and C) and non-thrombocytopenic (group A) dogs. A 389 bp product after nested PCR represents a positive sample (positive control, lane 4, 5, 7 and 9). Negative samples are present in Lanes 1, 2, 3, 6 and 8. No amplification was observed in the latter lanes.

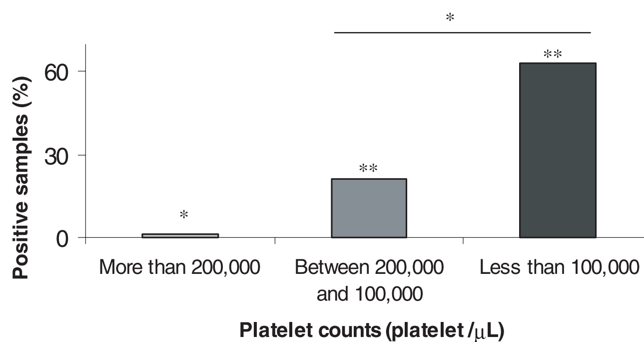

Figure 2. A graphical representation of the percentage of *E. canis* positive samples in each group. The * and ** represent statistical differences ($p < 0.001$) between the groups.

directing further diagnostic procedures. These results indicate an inverse relationship between the magnitude of thrombocytopenia and the prevalence of *E. canis* infection. The use of this information may suggest the stage of *E. canis* infection; however, one must rely on clinical findings and other diagnostic tests to make this determination. While having a normal platelet count does not completely rule out the presence of the disease [15], these results suggest that it is (very unlikely) less likely that dogs with normal platelet counts be infected with *E. canis* since only 1.4% of non-thrombocytopenic animals was positive.

The inverse relation of positive PCR results and the platelet count found was due to different thrombocytopenia etiologies in the different phases of EMC, which leads to different intensities. Dogs in the subclinical phase usually show mild thrombocytopenia [13, 15], however, even though dogs in the acute phase may show mild thrombocytopenia, they are most likely to show severe thrombocytopenia as dogs in the chronic phase [15].

Despite a variety of clinical and hematologic abnormalities identified in dogs infected with *E. canis*, a definitive diagnosis can be challenging. Diagnosis is usually made on the basis of a combination of clinical signs, hematologic abnormalities and serologic findings. Several methods exist to correctly diagnose a case of CME. Examination of a peripheral blood smear for the presence of *E. canis* morulae in mononuclear cells is a viable option of diagnosis. Morulae are most often found in the acute phase of the disease [2]. However, the sensitivity of this diagnostic method is poor since the morulae are found in only 4% of the positive cases [4]. An indirect fluorescence antibody assay is the most widely used test for the diagnosis of CME. This test can detect serum antibodies to the *E. canis* organism 7 days post-infection and the antibodies reach their peak at 80 days in an untreated dog. However, IFA is, at best an indicator of exposure to the pathogen. Following treatment, the antibody titer steadily falls and may become negative in 15–31 months [9]. In endemic areas, dogs can display a very high IgG titer to *E. canis* without displaying any clinical disease thereby causing an increase in false positive tests in these areas.

Also, there is the possibility of cross reactivity with *E. equi* and other *Ehrlichia* species [12]. Another diagnostic test, a polymerase chain reaction (PCR) for the *E. canis* 16S rRNA particle has been shown to be a sensitive method for the detection of acute *E. canis* infection in dogs. This test may however be time consuming, requires a specific level of technical expertise and is not readily available in most diagnostic laboratories. Therefore, we propose the use of the screening test through platelet counts before further diagnostic testing is performed. This approach may reduce the difficulties inherited with obtaining a definitive diagnosis and it may also minimize unnecessary and costly tests.

For this study, a nested-PCR assay for the presence of the *E. canis* organism in the blood was used to provide a definitive diagnosis. It has been shown that use of the nested-PCR for the diagnosis of *E. canis* is a hundred times more sensitive than one step PCR. It has been shown to be very specific, since the possibility of false positives by reaction with *E. chaffensis*, *E. muris*, *Neorickettsia helminthoeca* and the SF agent was tested and no false positive results were found [17]. It is recognized that this assay does have limitations in obtaining a definitive diagnosis as do other tests. Harrus et al. [7], found a lower sensitivity of the nested-PCR technique when using blood samples than when using splenic aspirates to the diagnosis of *E. canis* infection. However, performing complete blood count as a screening test using anticoagulated blood prior to PCR provides an easier method of sample collection.

In this study, 30.9% of the samples tested were positive by nested-PCR for the presence of *E. canis*. Other similar studies have shown a 17% positive rate in non-endemic regions and 44% in endemic areas in the USA, using the same technique [17]. It is likely that Botucatu is an endemic area. The weather conditions in São Paulo, 240 km from Botucatu, during the same period in which the samples were collected, were very rainy (142 mm of precipitation) and warm (max. 27 °C, min. 19 °C and average 23 °C). Although the cities are distant from each other, the climatic conditions are very similar. These weather conditions are ideal for the vector and since most dogs are frequently kept outdoors during these conditions, the probability of contact with the vector is most likely increased.

Numerous diseases can result in thrombocytopenia. These include immune-mediated thrombocytopenia, neoplastic processes, inflammatory diseases or other infectious agents [5]. None of these diseases can be eliminated from consideration once a thrombocytopenia is identified, even in an endemic area. However, based on the results presented here, consideration of *E. canis* infection should be routinely included in the

differential diagnosis when a thrombocytopenic dog is identified in an endemic area. We suggest that dogs with severe thrombocytopenia are good candidates for *E. canis* infection especially in an endemic area.

Screening tests are often very simple and inexpensive to use in a clinical setting. The results of this study suggest that the use of platelet counts as a screening test is a worthwhile clinical tool that should be employed in endemic areas to assist in determining further diagnostic procedures.

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