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Ehrlichia canis detection in dogs from Várzea Grande: a comparative analysis of blood and bone marrow samples

Detecção de *Ehrlichia canis* em cães domiciliados em Várzea Grande: análise comparativa entre amostras de sangue e medula óssea

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ABSTRACT

*The objective of this study was to compare the DNA detection of **Ehrlichia canis** in blood and bone marrow to determine the prevalence of the agent in Várzea Grande, Mato Grosso. Blood samples and bone marrow from 80 dogs of both sexes, different breeds and age, were collected and processed for a cross-sectional study performed using nested PCR. Of the 80 dogs, 61 (76.3%) had **E. canis** DNA in one of the samples. The buffy coat was positive in 42 dogs (52.5%) and the bone marrow was positive in 33 (41.3%). There was no significant association between the positive biological samples of either the buffy coat or bone marrow and the presence or absence of clinical signs (P=0.49). No risk factor was associated with infection in the studied area. The bone marrow samples were efficient for the molecular diagnosis of canine ehrlichiosis, particularly when there was a negative blood sample, although infection was present.*

Key words: *canine ehrlichiosis, diagnosis, nested PCR, biological samples.*

RESUMO

*Este trabalho teve por objetivo comparar a detecção de DNA de **Ehrlichia canis** em amostras de sangue e medula óssea, além de determinar a ocorrência do agente em Várzea Grande, Mato Grosso. Amostras de sangue e medula óssea de 80 cães, de ambos os sexos, diferentes raças e idade, foram coletados em estudo seccional e processados para realização de nested PCR. Dos 80 cães, 61 (76,3%) apresentaram DNA de **E. canis** em uma das amostras pesquisadas. A capa leucocitária foi positiva em 42 (52,5%) e a medula óssea em 33 (41,3%). Não foi observada associação significativa com a positividade das amostras biológicas, sangue ou medula óssea, e a presença ou ausência de sinais clínicos (P=0,49). Nenhum fator de risco foi associado à infecção na área pesquisada. A amostra de medula óssea mostrou-se bom sítio para o diagnóstico molecular da erliquiose canina, principalmente quando da infecção com negatividade da amostra sanguínea.*

Palavras-chave: *erliquiose canina, diagnóstico, nested PCR, amostra biológica.*

INTRODUCTION

Ehrlichia canis, the etiological agent of canine monocytic ehrlichiosis (CME), is a bacteria distributed worldwide that may cause lethal disease in dogs (AGUIAR et al., 2015). The pathogenesis of the disease involves an incubation period of 8 to 20 days, followed by an acute, subclinical and sometimes chronic phase (HARRUS & WNER, 2011). Normally, during the acute phase, infected dogs recover spontaneously. However, when they enter the subclinical stage, the dogs remain infected for longer periods. At this stage, dogs do not eliminate the agent from the body, and they develop the chronic phase of the disease, characterised by bone marrow suppression and bleeding, followed by death (WANER & HARRUS, 2013).

Because the clinical signs associated with the disease are nonspecific, clinical diagnosis is difficult. Therefore, a laboratory diagnosis of infections caused by ***E. canis*** morulae is performed using the visualisation of mononuclear cells, the detection of

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antibodies using serological techniques and molecular analysis using PCR (TANIKAWA et al., 2013).

The introduction of molecular techniques for the detection of ***E. canis*** has allowed for the rapid, sensitive and specific diagnosis of acute and chronic phases of the disease. Various PCR techniques can be used, such as *nested* PCR (OLIVEIRA et al., 2009), RFLP-PCR (restriction fragment length polymorphism) and real-time PCR (BANETH et al., 2009). Several target genes, including p28, p30, dsb, and VirB9, and PCR for the 16S rRNA gene and p30 are the most commonly used targets (HARRUS & WANER, 2011). Different biological sites can also be used (WANER & HARRUS, 2013). Animal health and CME is of global importance. The objective of this study was to compare the presence of ***E. canis*** DNA in the blood and bone marrow of dogs, as well as to determine the occurrence of ***E. canis*** in Várzea Grande, Mato Grosso.

MATERIALS AND METHODS

Animals and study area

Dogs in this study were obtained from a cross-sectional study for canine visceral leishmaniasis in the municipality of Várzea Grande, Mato Grosso, the neighbourhoods of São Matheus, Jardim Eldorado and Parque Sabia, coordinates 15º57'55" S 54º58'06" W. The survey was conducted by home visits, considering one residence for every five, totalling 521 dogs. The bone marrow was obtained from approximately 10% of the population studied. Dogs of all ages, both sexes and different breeds were included in this study with prior permission of the owner. The dogs were clinically evaluated for the presence of clinical signs of infection with ***E. canis***, such as apathy, anorexia, weight loss, lymphadenopathy, hepatomegaly, splenomegaly and ophthalmopathy and the clinical and epidemiological characteristics of the registered disease.

With the consent of the owners, the dogs were mechanically restrained and underwent sedation with ketamine hydrochloride (10mg kg^{-1}) and acepromazine (0.2mg kg^{1}). Approximately 5ml blood was collected by cephalic or jugular puncture into tubes containing anticoagulant for the recovery of the buffy coat by centrifugation. Bone marrow samples (0.5mL) were obtained via aspiration of the sternum manubrium and were stored in microtubes containing anticoagulant after prior asepsis and local anaesthesia. The biological specimens were stored at -20° until use.

DNA extraction and PCR

Extraction of DNA samples was performed using phenol/chloroform/isoamyl alcohol according to GOMES et al. (2007). ***E. canis*** DNA detection was performed using *nested* PCR. The primers used for the first amplification step were as follows: ECC (5'-AGAACGAACGCTGGCGGCAAGC-3') and ECB (5'-3'CGTATTACCGCGGCTGCTGGCA-3'). Primers for the second stage were ECAN (5'-CAATTATTTATAGCCTCTGGCTATAGGA-3') and HE3(5'-TATAGGTACCGTCATTATCTTCCCTAT-3'). These primers amplify fragments of 458 and 398 bp, respectively, of the 16S rRNA gene (MURPHY et al., 1998). Positive control was (dog 3577) an animal positive for ***E. canis***, and a negative control (DNA-free reaction) was also included in all PCR experiments. Amplified products were fractionated by agarose gel electrophoresis, stained with 1.5% gel Red and visualised transilluminator (UV-300 nm). To verify that no ***Leishmania*** DNA was amplified, the primers used were tested using the DNA reference strain ***L. (L.) infantum*** (MHOM/BR/1974/PP75), and no nonspecific amplification was observed. To confirm the species, eight (10%) samples were sequenced at the Sanger Sequencing facility (Applied Biosystems® Genetic Analysis, Foster City, CA) according to the manufacturer's recommendations.

Statistical analysis

Data were transferred to a database and analysed with the software Epi Info 3.3.2 (CDC, Atlanta, GA, USA) using Fisher's exact or chi-square tests to assess the association between independent variables, the presence of ***E. canis*** DNA positive results, the differences in the frequency for each clinical sample and the comparison of the clinical status of dogs at the 5% significance level.

RESULTS

Of the 80 dogs surveyed, 61 (76.3%) had ***E. canis*** DNA in one of the surveyed samples. No association was observed between gender, age, race, origin of dogs, free access to the street, or the presence of ticks (Table 1). In the analysis of biological samples, the buffy coat were positive in 42 (52.5%) and the bone marrow was positive in 33 (41.3%) of 80 dogs. Of the positive dogs, 14 (17.5%) showed DNA amplification of ***E. canis*** in both samples. The PCR products of eight dogs (10%) were sequenced, resulting in DNA sequences 100% identical to the sequences of ***E. canis*** in GenBank (accession numbers KP844663.1, KJ995842.1, KF972452.1, and JX118827.1).

Of all the dogs, 29 (36.3%) were asymptomatic, and 51 (63.8%) showed some clinical signs consistent with infection by ***E. canis***, of which, 21 (72.4%) and 40 (78.4%), respectively, were positive by PCR, with no statistically significant difference (P=0.73). The primary clinical signs

Table 1 - Epidemiological factors associated with positive PCR for ***Ehrlichia canis*** in a population of pet dogs in Várzea Grande, Mato Grosso.

Variables	---------Dogs--------		Análise Univariada	
	Total	Positive (%)	*P*	OR (CI95%)
Gender				
Male	46	37(80.4%)	0.30	0.58
Female	34	24(70.6%)		(0.18-1.75)
Breed				
SRD*	76	58(76.3%)	0.67	0.93
CRD*	04	03(75%)		(0.10-2.11)
Age groups (year)				
Indefinite age	18	15(83.3%)		
< 1	10	06(60%)		
1-3	17	15(88.2%)	0.35	------
3-6	21	16(76.2%)		
>6	14	9(64.3%)		
Access to the street				
Yes	33	24(72.7%)	0.53	0.72
No	47	37(78.7%)		(0.23-2.06)
Ticks				
Yes	41	30(73.2%)	0.50	0.70
No	39	31(79.5%)		(0.22-1.91)

*SRD - Without defined Race; *CRD - With defined Race.

observed were apathy, weight loss, lymphadenopathy, splenomegaly, hepatomegaly and ophthalmopathies.

For clinical the presence or absence of clinical signs, there was no significant association between the positivity of the biological samples, buffy coat and bone marrow (Table 2). There was no significant association between the two types of biological samples within the asymptomatic dog group (P=0.24) and symptomatic group (P=0.49).

DISCUSSION

The high incidence of infection with ***E. canis*** found in this study is consistent with data obtained in different regions of Brazil (DAGNONE et al., 2009; TANIKAWA et al., 2013) and Mato Grosso (MELO et al., 2011) based on serological analysis. In this study, nested PCR of buffy coat and bone marrow samples showed an occurrence of infection in 76% of dogs. This finding differs from that of WITTER et al. (2013), who analysed buffy coat samples and observed ***E. canis*** infection in 23.3% of dogs. The high occurrence can be related using two sites where bacteraemia is observed during different stages of the disease (MILONAKIS et al., 2003).

Direct exposure to the vector tick, ***R. sanguineus*** (CARLOS et al., 2011) and age (COSTA JUNIOR et al., 2007) were considered risk factors for infection with ***E. canis*** in other regions; however, these factors were not associated with infection in the dogs surveyed, as described by SILVA et al. (2012).

In epidemiological analyses, the blood sample is easily obtainable and provides good results (SILVA et al., 2012; SANTOS et al., 2013). However, the detection of the agent can decrease at this site with the progression of infection or the treatment of bacteraemia compared to other sites (HARRUS et al., 2004; BANETH et al., 2009). In this study, the highest percentage of DNA amplification in buffy coat indicates acute infection as demonstrated by BANETH et al. (2009), who analysed an experimental infection with ***E. canis*** in dogs in blood and spleen. However, determining the stage of infection in dogs naturally infected with canine ehrlichiosis is difficult because of the possible presence of similar acute and chronic infection clinical signs (HARRUS & WANER, 2011).

The absence of apparent clinical signs and the long duration of the subclinical stage may hinder the detection of infection (HARRUS & WANER, 2011; WANER & HARRUS, 2013), likely represented in this study by the asymptomatic group. HARRUS et al. (1998) observed the increased detection of ***E. canis*** in spleen samples in the subclinical stage, and in blood and bone marrow in the acute phase, whereas the number of positive animals detected by PCR using blood and spleen samples was similar (HARRUS et al., 2004). However, spleen samples are not routinely

Table 2 - DNA detection of ***E. canis*** using nested PCR in the buffy coat and bone marrow compared to clinical signs observed in dogs in Várzea Grande, Mato Grosso.

Clinic Analysis	----------Biological Samples----------					
	Negative + / (%)	Buffy Coat + / (%)	Bone Marrow + / (%)	Both + / (%)	χ^2	P
Asymptomatic	8 (10)	12 (15)	6 (7.5)	3 (3.8)	2.42	0.49
Symptomatic	11 (13.8)	16 (20)	13 (16.3)	11 (13.8)		
Total	19 (23.7)	28 (35)	19 (23.7)	14 (17.5)		

used in clinical practice because obtaining the samples is invasive (BANETH et al., 2009).

MYLONAKIS et al. (2004) and SIARKOU et al. (2007) detected ***E. canis*** DNA in bone marrow samples of 68.42% and 75% of surveyed dogs, respectively, during the chronic phase of infection. Despite no association between clinical signs and samples, there was a higher rate of observation of ***E. canis*** DNA in blood samples, independent of the analysed group. However, MOREIRA et al. (2005) identified a greater number of developmental forms of ***E. canis*** in the bone marrow compared to dog blood during the acute phase of the disease. MYLONAKIS et al. (2003) reported the occurrence of ***E. canis*** sequestration in the spleen during the subclinical stage and chronic disease as well as in the bone marrow during the chronic phase.

CONCLUSION

Infection with ***E. canis*** is highly prevalent in dogs in the city of Várzea Grande, Mato Grosso, and bone marrow is a good site for the molecular diagnosis of canine ehrlichiosis, particularly when infection is suspected despite of negative blood sample.

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BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

The Ethics Committee on Animal Use approved this study (CEUA - UFMT) Protocol 23108.018081/12-0.

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