Misdiagnosis of canine monocytic ehrlichiosis: why do we still risk animal lives?

Diagnóstico incorreto de erliquiose monocítica canina: por que ainda arriscamos vidas animais?

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ABSTRACT

Canine Monocytic Ehrlichiosis (CME) is a tick-borne disease caused by *Ehrlichia canis* that manifests as acute, chronic, or subclinical forms without specific clinical symptoms. This disease is diagnosed using clinical and laboratory findings (blood smears, molecular techniques, and serology). This study aimed to demonstrate the occurrence of false-positive results for *Ehrlichia* spp. in veterinary clinical practice. Seventy dogs with positive blood smears before treatment for *Ehrlichia* spp. subjected to doxycycline and imidazole treatment were analyzed using hematological examination, polymerase chain reaction (PCR), and indirect immunofluorescence assay. PCR analysis identified no samples positive for *E. canis* according to PCR analysis, while serological techniques showed a frequency of 51.4% in dogs with antibodies (IgG) against *Ehrlichia* spp. There was a correlation between hyperproteinemia and titers > 10,240. Nonspecific changes occurred in 24.3% (17/70) of the patients with CME, such as anemia, leukopenia, and thrombocytopenia. The results indicated that the blood count and blood smear analysis were insufficient for diagnosis and that positive serological results associated with hematological changes suggestive of ehrlichiosis in dogs can be incorrectly assigned by a veterinarian, putting animals at risk.

Keywords: Diagnosis. *Ehrlichia canis*. False-positive. Treatment.

RESUMO

A Erliquiose Monocítica Canina (EMC) é uma doença transmitida por carrapatos causada pela *Ehrlichia canis*, apresentando formas aguda, crônica ou subclínica, sem sintomatologia clínica específica. O diagnóstico da doença é baseado na associação entre achados clínicos e laboratoriais (esfregaços de sangue, técnicas moleculares e sorologia). O objetivo deste estudo foi demonstrar a ocorrência de resultados falso-positivos para *Ehrlichia* spp. na prática clínica veterinária. Neste contexto, 70 cães com esfregaços sanguíneos positivos, antes do tratamento, para *Ehrlichia* spp. submetidos ao tratamento com doxiciclina e/ou imizol foram analisados por exame hematológico, testados por reação em cadeia da polimerase (PCR) e por ensaio de imunofluorescência indireta. Não houve a detecção de amostras positivas para *E. canis* pela análise de PCR, enquanto as técnicas sorológicas mostraram uma frequência de 51,4% de cães com anticorpos (IgG) contra *Ehrlichia* spp. Houve correlação entre hiperproteinemia e títulos > 10.240. 24,3% (17/70) apresentaram alterações inespecíficas que ocorreram na EMC, como anemia, leucopenia e trombocitopenia. Os resultados indicaram que o hemograma e a análise do esfregaço sanguíneo não foram suficientes para completar o diagnóstico em cães. No entanto, resultados sorológicos positivos associados a alterações hematológicas sugestivas de erliquiose em cães podem ser erroneamente atribuídos pelo veterinário, o que pode colocar em risco a vida dos animais.

Palavras-chave: Diagnóstico. *Ehrlichia canis*. Falso-positivo. Tratamento.

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Introduction

Ehrlichiosis is a tick-borne disease caused by the intracellular bacteria belonging to the genus *Ehrlichia*. This disease is frequently observed in veterinary clinical practice and affects animals (*Ehrlichia canis*, the most common in Brazil) and humans (*Ehrlichia chaffeenis* mainly) (Openshaw & Swerdlow, 2007; Sousa et al., 2009).

In dogs, canine monocytic ehrlichiosis (CME) is commonly caused by infection with the gram-negative bacterium *E. canis* (Rickettsiales: *Anaplasmataceae*) (Albernaz et al., 2007). This bacterium develops structures compatible with morulae in the cytoplasm of mononuclear cells or neutrophils, manifesting in the acute phase, lasting 2-4 weeks (Sainz et al., 2015). This is followed by the subclinical or asymptomatic stage, which has no specific clinical signs and persists for 6-9 weeks. This is followed by the subclinical or asymptomatic phase, which has no apparent clinical signs, starts 6-9 weeks post-infection, and can persist for years. This chronic form has the worst prognosis due to the clinical signs' severity (Elias, 1991; Harrus & Waner, 2011; Sales et al., 2015; Aguiar et al., 2020).

Clinical signs and animal history are essential for guiding veterinarians in the diagnosis. The disease is generally characterized by reduced levels of red blood cell elements and thrombocytes, assessed using complementary examinations such as blood count and biochemical tests (Gaunt et al., 2010; Harrus & Waner, 2011). Therefore, diagnosis can be made through other techniques such as observing morulae in monocytes and lymphocytes in blood smears, detecting *E. canis* DNA using polymerase chain reaction (PCR), and serum analysis to detect antibodies against anti-*Ehrlichia* spp. using indirect immunofluorescence assay (IFA), enzyme-linked immunosorbent assay, and cellular cultivation. Clinically, veterinary medicine uses fast antibody and antigen test kits for qualitative diagnosis (Elias, 1991; Aguiar et al., 2007; Gaunt et al., 2010; Harrus & Waner, 2011; Costa et al., 2019).

According to Diniz & Aguiar (2022), the cytology of peripheral blood in the acute phase is more sensitive because of the larger pathogenic population. Visualization of morulae is difficult and time-consuming, even with high parasitemia, because the acute stages, when there are few morulae, can only occasionally be identified, resulting in many false negatives (Harrus & Waner, 2011). Furthermore, in the subclinical phase, only 4% or less of the animals present morulae (Fonseca et al., 2017). During the chronic phase, the pathological agents are barely detectable in the circulatory system.

This study aimed to demonstrate the occurrence of false positives for *Ehrlichia* spp. in routine veterinary clinical practice and to highlight the importance of the experience and training of technicians in correctly diagnosing *Ehrlichia* spp. morulae by blood smear.

Material and Methods

Animal selection and sample collection

Based on the statistical data and methods used to determine the sample size (Elias, 1991; Waner et al., 2000), 70 dogs with no distinction in breed, sex, or age were used in this study and sampled between June 2018 and May 2019 at a commercial laboratory in Jatai City, Goiás, Brazil. All procedures were approved by the Ethics Committee on the Use of Animals of the Jataí Region (CEUA/REJ/UFG - 009/2018). Additionally, the researchers preserved the identities of the dog owners and animals involved in this study according to international standards consistent with the ethical principles of the Brazilian College of Animal Experimentation (COBEA).

Blood samples (0.5 to 2 ml) were collected from the cephalic vein or jugular in a sterilized tube, and 10% universal anticoagulant (EDTA) was added to each sample. Another sample (3 ml), was collected in a tube with a cloth activator. An additional blood sample was obtained from the earlobe by puncturing with a small caliber (18 × 0.4 mm) for a blood smear to identify potential parasites.

After positive samples for *E. canis* were determined using blood smears (analyzed by the laboratory where the samples were collected), the blood and serum samples were sent, without a history of clinical signs, to the Virology and Rickettsiosis Laboratory at the Federal University of Mato Grosso to detect *E. canis* via PCR and IFA. All samples were collected simultaneously for use in different tests and before treatment.

Complete blood count, hematozoa research, and proteinogram

Blood counts, which consisted of total leukocytes, red cells, and hemoglobin, were measured using a hematological analyzer (Lab TestSDH Vet®). Platelet counts were performed manually using blood smears dyed with a commercial kit (Laborclin® Panoptic Kit) and analyzed using an optical microscope (Leica Microsystems®). Proteinograms were obtained using a reagent kit (LabTest®) according to the manufacturer's instructions in a biochemical analyzer (Spectrum Celer®). Average reference values used to compare hematological parameters were obtained and adapted from studies designed to define normal parameters (Lacerda, 2015).

Blood smears

A blood smear test was performed to screen for Ehrlichia infections. For this purpose, a blood smear was made in triplicate from venous blood smear (ESV) and kept in a tube with EDTA; from the ear tip (ESPO) was prepared immediately following collection, and from leukocyte capsule (ECL) (Kerr, 2003).

The blood smear technique produces a thin layer of blood on the surface of a glass slide for optical microscopy to observe the monolayer of cells (Fonseca et al., 2017).

Polymerase chain reaction (PCR) for E canis and Babesia

Blood samples were divided into 1.5 mL aliquots, EDTA was added, and the samples were stored at -20ºC until DNA extraction. The samples were defrosted using an extraction kit (Wizard® Genomic DNA Purification Kit, Promega Corporation) according to the manufacturer's instructions. PCR amplification was performed as described (Costa et al., 2019). The reaction targeted a 173 bp fragment of the *E. canis dsb* gene, with primers *dsb*-330 (5'-GATGATGTCTGAAGATATGAAACAAAT-3') and *dsb*-481 (5'-TGCTTGTAATGTAGTGCTGCAT-3'). A second protocol by Almeida et al. (2013) was adapted for the amplification of a 551 bp fragment of the *bab* gene, with primers *bab*-143-167 (5'-CCGTGCAATTGTAGGGCTAATACA-3') and *bab*-694-667 (5'-GCTTGAAACACTCTARTTTTCTCAAAG-3') used to discard the presence of another parasitosis, like Babesia, in case of error of diagnosis in the smeared blood. The PCR products were visualized on a 1.5% Agarose Gel after electrophoresis.

IFA

IFA was performed on confectioned slides of DH82 cells strain *E. canis* from São Paulo. Serum samples were diluted 1:40 in phosphate-buffered saline (pH 7.2) to detect antibodies against anti-*Ehrlichia* spp. (Aguiar et al., 2007).

Statistical analysis

The correlation between samples determined positive by IFA and hematological changes was conducted using the Mann-Whitney test in R (R Development Core Team, 2013) with a 95% confidence interval. The relative frequency of each data point was calculated by dividing the absolute frequency of each event by the total number of observations (70).

Results and Discussion

Of the 70 animals used in the study, 35 were male, and 35 were female. All were between 6 months and 10 years, and only four had a defined breed. None of the animals presented with ticks during collection, although 22.8% (16/70) had a history of infestation. All the samples were collected on the same day.

According to the laboratory, the Morulae of *Ehrlichia* spp. were detected in 100% of the blood smear samples (70/70), indicating that all animals were positive. In contrast, PCR did not identify positive *E. canis* and *Babesia* spp. samples, revealing the negation of parasitosis in the animals used in this study. All 70 animals were treated for *Ehrlichia* spp. with doxycycline, doxycycline, and imidazole, based on blood smears. The treatment was decided by the veterinarian who attended after obtaining the blood smear results. Therefore, this choice was not the responsibility of our study.

Blood counts showed that 62.8% (44/70) of the dogs had some hematological alterations, and 24.3% (17/70) had nonspecific changes that occurred in CME, such as anemia, leukopenia and thrombocytopenia (Table 1). Of the 20 animals with high anti-IgG antibody titers (> 10, 240), 15 presented with hyperproteinemia. The correlation between hyperproteinemia and titration was significant ($P < 0.05$). The presence of antibodies, as shown by IFA, verified the samples' reactivity of 51.4% (36/70). The endpoint titers varied between 1:40 and 1:655,360. Of the 36 animals that tested positive for IFA, only six presented with leukopenia, indicating no relationship between a positive IFA result and leukopenia.

Blood smears performed by the commercial laboratory showed that 100% of the animals were positive for *Ehrlichia* spp. However, the PCR results revealed showed no presence of *Ehrlichia* spp. DNA. PCR has been proven to be an efficient method for detecting *E. canis* during the acute phase of the disease, the same phase in which morulae are more often found (Waner et al., 2000; Ueno et al., 2009; Chung et al., 2021). The significant difference between the PCR and blood smear results was related to the technicians' experience interpreting blood smear examinations.

Table 1 – Frequencies of hematological changes present in the complete blood count of 70 dogs with suspected ehrlichiosis between June 2018 and May 2019 in Jatai, Goiás, Brazil

Hematological changes	Absolute	Relative Frequency (%)	Confidence Interval (%)*
Normocytic and normochromic anemia	9/70	12.86	10.28-29.66
Microcytic and normochromic anemia	4/70	5.71	5.07-21.28
Polycythemia	9/70	12.86	10.28-29.66
Leukocytosis	14/70	20.00	11.39-31.27
Eosinophilia	10/70	14.49	11.39-31.27
Neutrophilia	7/70	10.9	7.17-25.04
Lymphocytosis	1/70	1.43	0.04-7.70
Leukopenia	7/70	10.00	4.12-19.52
Neutropenia	2/70	2.90	0.35-10.08
Lymphopenia	4/70	5.71	1.58-13.99
Monocytopenia	1/70	1.43	0.04-7.70
Thrombocytosis	6/70	8.57	3.21-17.73
Thrombocytopenia	6/70	8.57	3.21-17.73
Hyperproteinemia	11/70	15.71	8.11-26.38
Hypoproteinemia	1/70	1.43	0.04-7.70

*Considered at a 95% confidence interval. No hematological data correlated with positive samples in the Indirect Fluorescent Antibody Technique ($P < 0.05$) using the Mann-Whitney test.

Smear analysis typically occurs in a small zone (smear tail) where a cell monolayer is found, and it is possible to visualize leukocytes in detail (Thrall et al., 2014). Some structures, such as platelets, lymphocytic azurophilic granules, phagocytosed nuclei, and artifacts from the preparation, such as morulae (Ehrlichia inclusions), can confuse technicians (Ueno et al., 2009; Harrus & Waner, 2011; Thrall et al., 2014; Rotondano et al., 2015; Diniz & Aguiar, 2022).

Inclusion bodies can be seen on canine monocytes in cases of infection by members of the *Anaplasmataceae* family and *Ehrlichia* (Kohn et al., 2011; Harrus & Waner, 2011). Furthermore, the morphological features of morulae make it impossible to identify the cell type (monocytes or granulocytes). *Ehrlichia canis* can infect monocytes, macrophages, and neutrophils (Diniz & Aguiar, 2022). Ramos et al. (2009) reported that inclusion bodies are primarily associated with cellular activation due to inflammation. As demonstrated in this study, the reduced sensitivity of blood smears compared with that of PCR (Elias, 1991; Arraga-Alvarado et al., 2014; Sales et al., 2015) confirms the importance of using a more sensitive technique in clinical practice.

Sales et al. (2015) compared parasite diagnostic techniques in 85 dogs. Although the study included animals in the acute phase, it still obtained a small proportion of positives (1.17%) owing to difficulties in technique execution and detection of infectious agents in the blood. These are essential factors to consider in veterinary clinics that do not have access to PCR tests to confirm the absence of parasitism.

In cases where the blood smear is negative, a veterinarian often subsequently makes a diagnosis using accessible and rapid tests. The most used in Brazil is the SNAP® 4Dx® (IDEXX Laboratories, Maine, USA), which detects antibodies for *Anaplasma* spp. (*A. platys*/*A. phagocytophilum*) and *Ehrlichia* spp. (*E. canis*/*E. ewingii*), *Borrelia burgdorferi*, and antigens of *Dirofilaria immitis*. Another commercial quick test that is less commonly used in Brazil is ImmunoComb ® (Biogal Galed Laboratories, Israel) (Dantas-Torres et al., 2018; Medeiros et al., 2020). However, positive results of immunochromatography or serological tests do not necessarily mean that the animal has a parasite because animals previously exposed to *Ehrlichia* spp. can have elevated antibodies for months or years after infection (Kaewmongkol et al., 2017; Medeiros et al., 2020). Furthermore, negative results in commercial tests do not negate the agent's presence, as animals can produce antibodies not detectable by the tests (O'Connor et al., 2006). Combining PCR with sorting tests, such as blood smears, quick tests, and hematology, can improve the accuracy of diagnosis (Medeiros et al., 2020)

Subclinical ehrlichiosis usually correlates with hematological alterations such as thrombocytopenia and anemia (Fonseca et al., 2017). In this study, 8.57% of the animals had thrombocytopenia; however, other infections or diseases that consume and destroy platelets, immunological deficiencies, and neoplasms can cause thrombocytopenia (Bai et al., 2017). As all the PCR results were negative, the thrombocytopenia was not related to the presence of *E. canis* in the acute phase. Previous studies have reported that PCR patients frequently observe thrombocytopenia, supporting our hypothesis (Frank & Breitschwerdt, 1999; Ueno et al., 2009; Fonseca et al., 2017).

Anemia in 18.6% of animals is associated with CME in endemic areas (Ueno et al., 2009; Paula et al., 2022). However, hematological and non-hematological diseases, such as infections, neoplasms, malnutrition, nutritional deficiencies, and kidney diseases, correlate with anemia (Latimer, 1997; Frank & Breitschwerdt, 1999; Lasta et al., 2013). If an animal is asymptomatic, anemia can be related to factors such as breed and age (Bai et al., 2017). The anemia detected in this study was not associated with *E. canis* as all PCR were negative.

Hyperproteinemia was observed in approximately 16% (11/70) of the animals, and most cases occurred because of an increase in globulins after high titration of the dogs (Lacerda, 2015). High levels of serum proteins have been reported in subjects with more gamma globulins, which are related to chronic immune stimulation. In the acute phase of illness, an increase in these parameters persists through the subclinical and chronic stages (Asgarali et al., 2012). Nevertheless, a previous study reported a decrease in serum protein levels and an increase in globulin levels in cases of liver damage (Parashar et al., 2016). An increase in protein levels during the acute phase has been previously reported (Harrus & Waner, 2011)

IFA revealed that 51.4% (36/70) of the samples were seropositive owing to a persistent immunogenic stimulus. This phenomenon has been observed in studies of dogs suspected of having ehrlichiosis (Harrus & Waner, 2011; Rufino et al., 2013). However, high antibodies are expected in symptomatic dogs. These results agree with those of previous studies in the central-western region of Brazil, where more than 50% of the animals had last contact with *E. canis* (Santos et al., 2013; Soares et al., 2017; Paula et al., 2022). However, the presence of anti-*E. canis* indicates that the animal had contact with the bacteria but not necessarily at the time of examination (Aguiar et al., 2007).

According to this study, 24.2% (17/70) of animals with hematological changes corresponded to markers thought to be indicative of CME in the acute phase based on complete blood counts (anemia, thrombocytopenia, and leukopenia), which would prompt veterinarians to prescribe unnecessary drugs when there is no evidence of circulating agents. Thus, the primary cause of these signs can be neglected and not appropriately diagnosed, resulting in the increased use of therapeutic measures without absolute necessity and the exposure of the patient to the collateral effects of medications, such as doxycycline and other antimicrobials used against Ehrlichiosis (Sales et al., 2015). Doxycycline treats gram-negative and gram-positive bacterial infections and is considered a broad-spectrum antibiotic.

When used for an extended period, it can cause vomiting, diarrhea, and bone deficiency, damage the development of young animals, and drive the growth of antimicrobial resistance (Mylonakis et al., 2019; Spinosa et al., 2022). As mentioned above, due to the clinical signs, all animals were unnecessarily treated by veterinary clinicians with medications for hemoparasitosis, including doxycycline and imidazole, even though they were not parasitized by *Ehrlichia* spp. or *Babesia* spp. This conduct was not part of our remit but was the veterinarian's choice.

From these results, it can be concluded that asymptomatic or non-specifically symptomatic dogs should be thoroughly evaluated for infectious agents such as *Ehrlichia* spp., as indicated by blood count and blood smear analysis. However, positive serological results associated with hematological changes suggestive of ehrlichiosis in dogs can be incorrectly diagnosed by a veterinarian, leading to the prescription of medication to treat parasitism and putting animals at risk because of the inappropriate use of medicines or misdiagnosis of alterations common to CME and other diseases.

Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Ethics Statement

All procedures were approved by the Ethics Committee on the Use of Animals of the Jataí Region (CEUA/REJ/UFG - 009/2018). Additionally, the identities of the dogs' owners and the animals involved in this study were preserved according to the international standards consistent with the animal experiment ethical principles of COBEA (Brazilian College of Animal Experimentation).

Acknowledgements

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