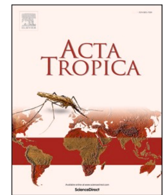
Acta Tropica 219 (2021) 105931

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica



# Serological evidence of *Ehrlichia minasensis* infection in Brazilian dogs

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A R T I C L E  I N F O

*Keywords:*
Ehrlichia canis
ELISA
IFA
canine
serology
Brazil

A B S T R A C T

*Ehrlichia* spp. are important tick-borne pathogens of animals in Brazil, and *Ehrlichia canis* is the most prevalent species infecting dogs. Moreover, *Ehrlichia minasensis* has also recently been identified as a novel ehrlichial agent that infects cattle in Brazil. The objective of this study was to determine whether dogs could be infected by *E. minasensis*. To investigate this possibility, sera (n = 429) collected from dogs in the Pantanal region were retrospectively analyzed for the presence of antibodies against *E. canis* and *E. minasensis*. Canine sera were screened by two isolates of *E. canis* in indirect immunofluorescence assay (IFA) and the majority (n = 298; 69.4%) had antibodies with endpoint titers ranging from 80 to 327,680. In order to further confirm *E. canis*-specific antibodies, IFA positive sera were analyzed by ELISA using *E. canis*-specific peptides (i.e. TRP19 and TRP36 US/BR/CR), which detected *E. canis* antibodies in 80.2% (239/298) of the dog sera. Fifty-nine (13.7%) samples had detectable antibodies to *E. canis* by IFA but were negative by *E. canis* peptide ELISA. These sera were then tested by *E. minasensis* IFA (Cuiaba strain) as antigen and 67.8% (40/59) were positive (titers ranging from 80 to 20,480). Eleven sera had antibody titers against *E. minasensis* at least two-fold higher than observed for *E. canis* and suggests that these dogs were previously infected with *E. minasensis*. The results of the present study suggest that multiple ehrlichial agents infect dogs in Brazil, which highlights the need to consider different

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https://doi.org/10.1016/j.actatropica.2021.105931
Received 23 December 2020; Received in revised form 12 April 2021; Accepted 13 April 2021
Available online 24 April 2021
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*Ehrlichia* spp. in Brazilian dogs, particularly in areas where dogs are frequently exposed to multiple tick species. This investigation is the first to provide serologic evidence of *E. minasensis* infection in dogs from Brazil.

## 1. Introduction

The genus *Ehrlichia* consists of important tick-borne pathogens of domestic and wild animals (Dumler et al., 2001; Cabezas-Cruz et al., 2012; Aguiar et al., 2013, 2014). In Brazil, *Ehrlichia canis* is widespread in the tropical areas of the country and it is one of the most important pathogens of dogs transmitted by *Rhipicephalus sanguineus* sensu lato (s. l.) ticks (Aguiar et al., 2007, 2013). Recently, a new *Ehrlichia* species, namely *Ehrlichia minasensis*, has been isolated from *Rhipicephalus microplus* ticks (Cabezas-Cruz et al., 2012, 2016) and cattle in Brazil (Aguiar et al., 2019a; André et al., 2020). In addition to cattle, *E. minasensis* has been detected in cervids (Lobanov et al., 2012), horses (Muraro et al., 2021a), and other mammals of the superorder Xenarthra (Calchi et al., 2020). The presence of *E. minasensis* in several tick species within the genera *Rhipicephalus* (Iweriebor et al., 2017), *Hyalomma* (Cicculli et al., 2019; Li et al., 2019), and *Haemaphysalis* (Li et al., 2019) suggests that the bacteria could be transmitted by several ticks. A molecular evolution analysis suggested that *E. minasensis* originated from a highly variable strain of *E. canis* and possibly for this reason these species are closely related (Cabezas-Cruz et al., 2014). Intriguingly, despite being closely related to *E. canis*, to date, *E. minasensis* has not been reported in dogs.

Detection of specific antibodies to ehrlichial pathogens is an essential tool for epidemiological studies, since direct detection of these agents in blood samples may result in false negative results due the low bacteria levels in the prolonged periods of non-bacteremic phase (Harrus et al., 2004). Although indirect immunofluorescence assays (IFA) are widely used for serological diagnosis of *Ehrlichia* spp., cross-reactions with antigens of other bacteria within the family Anaplasmataceae can often produce misleading results (Harrus and Waner, 2011). This problem has been overcome by the use of enzyme-linked immunosorbent assays (ELISA) using secreted proteins, TRP19 and TRP36, that are specific to *E. canis* (Yu et al., 2007; McBride et al., 2007; Zhang et al., 2008; Aguiar et al., 2016). The use of these methodologies may be useful to elucidate epidemiological aspects of *Ehrlichia* spp. infections, since seroprevalence for these agents is high among canines in Brazil (Doyle et al., 2006; Cárdenas et al., 2007; McBride et al., 2007; Vieira et al., 2011; Dantas-Torres et al., 2018, 2020).

Previous studies showed the presence of ixodid ticks and tick-borne pathogens in the Brazilian Pantanal region (Melo et al., 2011, 2015, 2016; Sacchi et al., 2012; Alves et al., 2014; Muraro et al., 2021b). *Ehrlichia minasensis* is one of these pathogens that was detected in cattle and *R. microplus* ticks in this region (Aguiar et al., 2014, 2019a; Carvalho et al., 2016, André et al., 2020). Dogs that inhabit farms in Pantanal are exposed to ticks and tick-borne agents (Melo et al., 2011). Despite the evidence of different *Ehrlichia* spp. circulating in the Pantanal, only *E. canis* has been detected in dogs and their ticks (Santos et al., 2013; Melo et al., 2016; Souza et al., 2017).

The circulation of ehrlichial agents demonstrated in the latest serological research in this area (Melo et al., 2011; Widmer et al., 2011; Barros et al., 2015) highlights the need to know more about pathogens that elicit these antibodies and obtain more epidemiological information about these pathogens transmitted by ticks. Considering the importance of *E. minasensis* in cattle from the Pantanal region and their close genetic relationship with *E. canis*, we surveyed dogs for serologic evidence of *E. minasensis* infection. *Ehrlichia minasensis* antigen produced following isolation in cell culture (Aguiar et al., 2019b) was used for the first time by IFA to determine the presence of antibodies against *E. minasensis* in dogs.

## 2. Materials and methods

### 2.1. Sample collection

This study included serum samples from 429 dogs sampled from 2009 to 2012 in four municipalities located in the Pantanal wetlands, namely: i) Poconé, Mato Grosso state (56° 37′ 22" W; 16° 15′ 24" S); ii) Santo Antônio de Leverger, Mato Grosso state (56° 04′ 47" W; 15° 51′ 47" S); iii) Barão de Melgaço, Mato Grosso state (55° 58′ 03" W; 16° 11′ 49" S); and iv) Corumbá, Mato Grosso do Sul state (57° 39′ 44"W; 19° 00′ 33"S). The samples from Poconé (n = 319) were previously evaluated by Melo et al. (2011). The remaining 110 samples were collected for the present work in Santo Antônio de Leverger, Barão de Melgaço and Corumbá. Blood samples were collected from dogs by jugular venipuncture and serum was obtained by centrifugation at 1,580 *g* for 5 minutes.

### 2.2. IFA

Anti-*Ehrlichia* spp. antibodies were screened by IFA using the São Paulo and Cuiaba#1 strains of *E. canis* with cut-off point at an initial dilution of 1:80 (Aguiar et al., 2007; Melo et al., 2011). All IFA positive sera were examined by ELISA using *E. canis*-specific peptides as described in the next section. In an attempt to verify the presence of antibodies against *E. minasensis*, all sera positive by IFA, in which *E. canis*-specific antibodies were not confirmed by ELISA, were screened by IFA using the Cuiabá strain of *E. minasensis* (Aguiar et al., 2019b) at an initial dilution of 1:80. The production of *E. minasensis*-infected antigen for IFA was performed similarly to that of *E. canis* (Aguiar et al., 2007; Melo et al., 2011). Considering the potential genetic and antigenic similarity between *E. canis* and *E. minasensis*, sera with antibody titers at least two-fold higher to *E. minasensis* were considered to be positive.

### 2.3. ELISA

Peptides corresponding to the tandem repeat regions of the TRP19 of *E. canis* (24-mer, HFTGPTF-SEVNLSEEEKMELQEVS) (McBride et al., 2007), the TRP36 from Brazil (BR) *E. canis* strain (24-mer, ASVVPEAEASVVPEAEASVVPEAE) (Aguiar et al., 2013), TRP36 from Costa Rica (CR) *E. canis* strain (28-mer, EASVVPAAEAPQPAQQTEDEFFSDGIEA) (Arroyave et al., 2020) and TRP36 from the United State (US) *E. canis* strain (18-mer, TEDSVSAPATEDSVSAPA) (Doyle et al., 2006) were synthetized (Bio-Synthesis Inc., Lewisville, TX). A peptide corresponding to the TRP36 C-terminal region from the *E. canis* Israeli strain (IS36-C-V, 15-mer, NPTGLKFLDLYTQLTL) (Zhang et al., 2008) was used as a negative peptide control.

The ELISA assay was performed as previously described (Luo et al., 2010; Aguiar et al., 2016). The optical density (OD) was determined in a microplate reader (VersaMax; Molecular Devices, Sunnyvale, CA), and data was analyzed by using SoftMax Pro v4.0 (Molecular Devices). The OD values ($A_{650}$) of each sample represent the mean of three technical replicates after subtracting the OD value of the non-reactive negative control peptide (i.e., IS36-C-V). A positive sample threshold was set at >0.150 OD units above the absorbance of the negative control. Dogs were considered positive for *E. canis* antibody if they reacted with at least one *E. canis*-specific peptide by ELISA.

## 3. Results

### 3.1. Dog sera reaction to E. canis antigens

Reaction for both *E. canis* strains in the IFA assays were observed in 298 samples (69.4%) and 80.2 % (239/298) of dog sera had antibodies that reacted with one or both TRP19/TRP36 peptides of the *E. canis* strains US, BR and CR. Fifty-nine (13.7%) dogs had antibodies that reacted with one or both *E. canis*-IFA antigens, but were negative by *E. canis*-specific TRP ELISAs. Endpoint *E. canis*-IFA titers ranged from 80 to 327,680.

### 3.2. Dog sera reaction to E. minasensis antigens

*E. canis*-positive IFA sera (n=59) were tested using *E. minasensis* IFA and 40 (67.8%) were positive with endpoint titers ranging from 80 to 20,480. Positive samples showed the following titers: 80 (6 dogs); 160 (3); 320 (2); 640 (11), 1,280 (5); 2,560 (6); 5120 (5); 10240 (1) and 20,480 (1). Eleven samples showed antibody titers against *E. minasensis* antigen at least two-fold higher than *E. canis* antigen (Table 1).

## 4. Discussion

A previous study reported dogs with antibodies against *Ehrlichia* spp. in the northern Pantanal (Melo et al., 2011). The higher presence of positive dogs in the IFA than species-specific *Ehrlichia* ELISA assays reinforces the limitations of IFA for identifying specific agents, because of antigenic cross-reactivity that is well documented (Harrus and Waner, 2011). In order to enhance the screening of ehrlichial antibodies two distinct isolates of *E. canis* were used by IFA. The use of TRP19 and TRP36 proteins of *E. canis* on serological assays improve sensitivity to detect *E. canis*-specific antibodies (Doyle et al., 2006; Cárdenas et al., 2007; McBride et al., 2007; Zhang et al., 2008; Aguiar et al., 2013). ELISA results confirmed the presence of *E. canis*-specific antibodies in 80.2% (239/298) of the samples that were positive to *E. canis* by IFA. The ELISAs using TRP synthetic antigens were standardized as described by Aguiar et al. (2016), using serum samples from dogs experimentally infected by *E. canis* and *Ehrlichia chaffeensis* to demonstrate the specificity of the assay. In addition, a previous report showed that sera from dogs naturally infected with *E. canis* reacted against epitopes present in the TRP synthetic peptides used in this study (Doyle et al., 2006). The presence of ehrlichial pathogens in the canine population used in this study have been assessed using different genetic targets and methodologies (i.e., PCR, hemi-nested PCR and quantitative PCR) and only *E. canis* DNA was detected (Santos et al., 2013; Melo et al., 2016). However, this is the first study assessing the exposure of dogs to *E. minasensis* infection by means of serological tests.

**Table 1**
Antibody titers against *Ehrlichia* spp. by IFA in 40 dog sera non-reactive to *E. canis*-specific peptides (ELISA negative).

| Sample | Environment | IFA titers | | |
|---|---|---|---|---|
| | | *E. canis* São Paulo | *E. canis* Cuiabá#1 | *E. minasensis* |
| U98 | urban | 160 | 40,960 | 5,120 |
| U126 | | 160 | 320 | 640* |
| U128 | | 160 | 640 | 5,120* |
| U137 | | 160 | 320 | 80 |
| R11 | rural | 320 | 80 | 80 |
| R12 | | 640 | 640 | 1,280* |
| R17 | | 320 | 320 | 640* |
| R22 | | 320 | 640 | 80 |
| R25 | | 5,120 | 81,920 | 2,560 |
| R28 | | 320 | 1,280 | 80 |
| R30 | | 1,280 | 1,280 | 640 |
| R32 | | 2,560 | 640 | 320 |
| R62 | | 81,920 | 20,480 | 10,240 |
| R69 | | 1,280 | 10,240 | 5,120 |
| R73 | | <80 | 320 | 1,280* |
| R104 | | 5,120 | 1,280 | 2,560 |
| R106 | | 2,560 | <80 | 640 |
| R130 | | 320 | 640 | 80 |
| R147 | | 640 | 320 | 640 |
| R151 | | 160 | 320 | 640* |
| R154 | | 640 | 2,560 | 640 |
| R157 | | 5,120 | 5,120 | 5,120 |
| Cb 2 | rural | 640 | 640 | 1,280* |
| Cb 6 | | 320 | 160 | 320 |
| Cb 7 | | 640 | 5,120 | 1,280 |
| Cb10 | | 80 | 160 | 640* |
| Cb 12 | | 640 | 2,560 | 2,560 |
| Cb13 | | 640 | 2,560 | 2,560 |
| Cb 14 | | 640 | 640 | 2,560* |
| Cb 15 | | 160 | <80 | 1,280* |
| MT 6 | rural | 640 | 320 | 160 |
| MT 11 | | 1,280 | 640 | 160 |
| MT 16 | | 2,560 | 640 | 1,280 |
| MT 17 | | 2,560 | 1,280 | 5,120* |
| MT 19 | | 2,560 | 640 | 2,560 |
| MT 20 | | 640 | 320 | 640 |
| MT 25 | | 320 | 160 | 160 |
| Br 5 | rural | 1,280 | 2,560 | 640 |
| JL 24 | rural | 640 | <80 | 80 |
| JL 28 | | 10,240 | 20,480 | 20,480 |

*Antibody titers against *E. minasensis* antigen at least two-fold higher than *E. canis* antigen.

Antibodies reacting to *E. minasensis* antigen in IFA were found in most (67.8%) of the IFA-positive samples but negative for specific *E. canis*-specific antigens (TRP19 and TRP36) by ELISA. This result does not confirm infection by *E. minasensis* since cross reactive antibodies among *Ehrlichia* spp. are well-documented. However, these results when analyzed in conjunction with the *E. canis*-specific ELISA provides evidence that antibodies in some of these dogs were stimulated by other *Ehrlichia* spp., possibly by *E. minasensis*. For example, 11 dogs were seronegative in ELISA assays with *E. canis*-specific antigens but had antibody titers of *E. minasensis* two-fold higher than *E. canis* in IFA. Furthermore, from these dogs, at least five had antibody titers four-fold higher to *E. minasensis* than to *E. canis*. Evaluating antibody titers to homologous and heterologous antigens to provide evidence of the possible infecting agent involved has been previously used (Saito et al., 2008; Melo et al., 2011). Thus, it appears that these dogs were likely exposed to *E. minasensis*, supporting further investigation on *E. minasensis* infections in dogs. Furthermore, as the antibody titers to *E. canis* and *E. minasensis* antigens did not allow differentiation of the two agents in several samples, the possibility of co-infections cannot be ruled out. However, it is important to mention that reports of co-infection with different species of the genus *Ehrlichia* in dogs are extremely rare, which may be mainly due to the antigenic proximity of the agents, morphological similarity and leukocyte tropism (Popov et al., 1998; Aguiar et al., 2020). For example, there is a report of co-infection between *E. canis* and *E. chaffeensis* in dogs in Venezuela that was elucidated by isolation and molecular methods (Gutiérrez et al., 2008).

Sera from 19 dogs (4.4%) that were *E. canis*-IFA positive, but *E. canis*-peptide-ELISA and *E. minasensis*-IFA negative could be attributed to another *Ehrlichia* spp. In Brazil, in addition to the isolation of *E. canis* (Aguiar et al., 2013) and *E. minasensis* (Aguiar et al., 2019a), molecular detection of *E. chaffeensis* (Machado et al., 2006; Sacchi et al., 2012) and other possible native *Ehrlichia* species have also been described in wild animals (Widmer et al., 2011; Almeida et al., 2013; Soares et al., 2017; Lopez et al., 2018, André, 2018). Our results suggest that another *Ehrlichia* is circulating in the Brazilian Pantanal, since in other areas of this ecosystem (northern Argentina) species closely related to *E. chaffeensis* and *E. ruminantium* have been detected in ticks associated to wild and domestic dogs (Cicuttin et al., 2017, 2020; Eberhardt et al., 2020).

## 5. Conclusion

The present study provides important epidemiological data for the

occurrence of ehrlichial agents in dogs from the Brazilian Pantanal. Analysis of the canine serum bank against different antigens reinforces that most ehrlichial infections in dogs in Pantanal are caused by *E. canis*. However, for the first time in Brazil, we provide serological evidence for the presence of *E. minasensis* in dogs. The results of the present study demonstrate the need to use different ehrlichial antigens to search for antibodies against ehrlichial agents in dogs in Brazil, particularly in areas where dogs are frequently exposed to different tick species.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Ethical approval

This study was approved by the Committee on Animal Research Bioethics UFMT under protocol number 23108.019742/09-9.

## Declaration of interests

The authors declare that they agree with the submission of this paper.

## CRediT authorship contribution statement

**Andréia Lima Tomé Melo:** Conceptualization, Investigation, Writing - original draft, Writing - review & editing. **Tian Luo:** Investigation. **Xiaofeng Zhang:** Investigation. **Lívia Saab Muraro:** Investigation. **Nathalia Assis Pereira:** Investigation. **Alejandro Cabezas-Cruz:** Writing - review & editing. **Filipe Dantas-Torres:** Writing - review & editing. **Jere W. McBride:** Resources, Supervision, Writing - review & editing. **Daniel Moura de Aguiar:** Conceptualization, Resources, Supervision, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing.

## Acknowledgments

We thank to J.T. de Melo and E.C.L.T. de Melo for their technical support and Estância Ecológica SESC Pantanal for logistical support. This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) (1913/2011), Fundação de Amparo à pesquisa do Estado de Mato Grosso (FAPEMAT) (263287/2010) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (472206/2011-7). We thank CAPES and CNPq for the scholarships of A. L.T. Melo. F. Dantas-Torres and D.M. Aguiar are also recipients of a research fellowship from CNPq (respective grants number: 313118/2018-3 and 303677/2018-0). D.M. UMR BIPAR is supported by the French Government's Investissement d'Avenir program, Laboratoire d'Excellence "Integrative Biology of Emerging Infectious Diseases" (grant no. ANR-10-LABX-62-IBEID). Aguiar thanks God for his life and for his work in veterinary medicine.

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