Archives of Veterinary Science **ISSN 1517-784X**
v.26, n.2, p. 65-78, 2021 ww.ser.ufpr.br/veterinary

MOLECULAR DETECTION AND SEROPREVALENCE OF *Ehrlichia* sp. IN DAIRY CATTLE FROM BRAZIL'S WESTERN AMAZON REGION

(Detecção molecular e soroprevalência de *Ehrlichia* sp. em bovinos leiteiros da Amazônia ocidental, Brasil)

Rute Witter[1,4], Andréia Lima Tomé Melo[2], Thábata dos Anjos Pacheco[3,4], Valéria Dutra[4], Luciano Nakazato[4], Cristiane Silva Chitarra[4], Anderson Castro Soares de Oliveira[4], Daniel Moura de Aguiar[4], Richard Campos Pacheco[4] http://orcid.org/0000-0002-7135-1516

[1]Instituto Federal de Rondônia-IFRO, campus Jaru, Jaru, RO, Brasil, [2]Programa de Pós-Graduação em Biociência Animal, Universidade de Cuiabá, Cuiabá, MT, Brasil, [3]Instituto Federal de Rondônia-IFRO, Campus Cacoal, Cacoal, RO, Brasil, [4]Programa de Pós-Graduação em Ciências Veterinárias, Faculdade de Medicina Veterinária, Universidade Federal de Mato Grosso, Cuiabá, MT, Brasil.

*Corresponding author: richard@ufmt.br

Editora: Julia Arantes Galvão

ABSTRACT - *Ehrlichia minasensis*, a novel species of *Ehrlichia* that is closely related to *E. canis,* is known to infect cattle and deer in Canada. This rickettsial bacterium was isolated from *Rhipicephalus (Boophilus) microplus* ticks and from dairy and beef cattle in southeast and midwestern Brazil, respectively. The purpose of this study was to determine the seroprevalence and perform molecular detection of *Ehrlichia* sp. in dairy cattle in the northern region of Brazil. The study was conducted in the municipality of Ji-Paraná, located in the state of Rondônia in the western Brazilian Amazon region. Blood and serum samples were obtained between the dates September 2012 and November 2013 from dairy cows (≥ 24 months old) in 64 farms. The blood samples were subjected to polymerase chain reaction (PCR) to amplify a fragment of the *Ehrlichia* sp. *dsb* gene, and the levels of *Ehrlichia* sp. antibodies were measured by the indirect immunofluorescence assay (IFA). A total of 15 of 610 (2.45%; 95% CI: 1.04-3.86%) blood samples tested positive for ehrlichial infection based on the detection of the *Ehrlichia* sp. *dsb* gene. Sequencing of PCR amplicons from samples confirmed that the amplified partial *dsb* gene (~295 base pairs) sequence represented *E. minasensis*. Anti-*Ehrlichia* sp. antibodies were detected in 178 cows (53.96%; 95% CI: 46.63–61.29%). Endpoint titers ranged from 40 to 5,120. However, positive results derived from this assay should be interpreted with caution. Among the variables analyzed using IFA, the number of cows aged 24 months or greater was statistically significant (p = 0.0103), and hers of approximately 51-100 cows were more likely to be infected with *Ehrlichia* sp. Although the animals do not show clinical disease, the chronic character of the infection can lead to decrease in productivity.

Received in 04/16/2020
Approved in 06/29/2020

Key words: antibodies; *dsb* gene; *Ehrlichia minasensis*.

RESUMO - *Ehrlichia minasensis*, uma espécie de *Ehrlichia* que está intimamente relacionada a *E. canis*, é conhecida por infectar bovinos e veados no Canadá. Esta bactéria rickettsial foi isolada de carrapatos *Rhipicephalus* (*Boophilus*) *microplus* e de bovino leiteiro do Sudeste e Centro-Oeste do Brasil, respectivamente. O objetivo deste estudo foi determinar a soroprevalência e realizar a detecção molecular de *Ehrlichia* sp. em bovinos leiteiros da região Norte do Brasil. O estudo foi realizado no município de Ji-Paraná, localizado no estado de Rondônia, região oeste da Amazônia brasileira. Amostras de sangue e soro foram obtidas entre setembro de 2012 a novembro de 2013 de vacas leiteiras (≥ 24 meses) em 64 fazendas. As amostras de sangue foram submetidas à reação em cadeia pela polimerase (PCR) para amplificar um fragmento do gene *dsb* de *Ehrlichia* sp. e os níveis de anticorpos de *Ehrlichia* sp. foram avaliados pelo teste de imunofluorescência indireta (IFI). Um total de 15 de 610 (2,45%; IC 95%: 1,04-3,86%) amostras de sangue apresentaram resultado positivo com base na detecção do gene *dsb* de *Ehrlichia* sp. O sequenciamento do produto da PCR confirmou que a sequência parcial amplificada do gene *dsb* (~ 295 pares de base) representava *E. minasensis*. Anticorpos anti-*Ehrlichia* sp. foram detectados em 178 animais (53,96%; IC 95%: 46,63-61,29%). Os títulos dos anticorpos variaram entre 40 e 5.120. No entanto, resultados positivos deste estudo devem ser interpretados com cautela. Entre as variáveis analisadas usando IFI, o número de vacas ≥ 24 meses foi estatisticamente significativo (p= 0,0103), e as propriedades com 51 a 100 vacas apresentaram maior probabilidade de infecção por *Ehrlichia* sp. Embora os animais não apresentem doença clínica, o caráter crônico da infecção pode levar à diminuição da produtividade.
Palavras-chave - anticorpos; gene *dsb*; *Ehrlichia minasensis*.

INTRODUCTION

Ehrlichia species are tick-transmitted gram-negative obligate intracellular bacteria that infect mature and immature hematopoietic cells and more importantly, the mononuclear phagocyte system (monocyte and macrophages), myeloid cells (neutrophils) and endothelial cells of the mammalian body (Dumler et al., 2001). Ehrlichiosis is an infectious disease of mammals that is caused by different species of the genus *Ehrlichia*. Currently, this genus comprises of the species *E. canis, E. chaffeensis, E. muris, E. ewingii, E. ruminantium,* and *E. minasensis* (Dumler et al., 2001; Cabezas-Cruz et al., 2016), that

are responsible for emerging zoonoses in humans (Paddock and Childs, 2003; Aguiar et al., 2014).

Genotypes nearly resembling *E. ruminantium* and *E. muris* have been identified in ruminants (Loftis et al., 2008) and humans in the United States (Pritt et al., 2011), respectively. In addition, an *Ehrlichia* genotype phylogenetically close to *E. canis* was also sighted infecting cattle and deer (*Odocoileus hemionus*) in Canada (Gajadhar et al., 2010, Lobanov et al., 2012). A previously unidentified ehrlichial species was isolated from *Rhipicephalus* (*Boophilus*) *microplus* female ticks collected in Brazil (Cabezas-Cruz et al., 2012; Zweygarth et al., 2013). Molecular characterization of this agent through cell culture and electron microscopy was performed for identification and named this new species as *Ehrlichia mineirensis* (Zweygarth et al., 2013; Cabezas-Cruz et al., 2016). In 2014, *E. minasensis* was isolated from dairy and beef cattle in midwestern Brazil, and discovered to be the cause of clinical ehrlichiosis in an experimentally infected calf (Aguiar et al., 2014). After genetic characterization of these isolates from their 16S rRNA, it was observed that the *Ehrlichia* genotypes detected from the cattle and deer of Canada (Gajadhar et al., 2010), and from *R.* (*B.*) *microplus* (Cabezas-Cruz et al., 2012, 2016; Carvalho et al., 2016) and cattle in Brazil belong to the same species of *Ehrlichia* (Aguiar et al., 2014, 2019a). This new species of *Ehrlichia* may be related to the former *Ehrlichia bovis* (Massard, 1984) detected from the monocytes of an infected dairy cattle in the states of Rio de Janeiro and Minas Gerais, Brazil. Currently, *E. minasensis* has a wide geographical distribution, already being reported in Ethiopia (Hailemariam et al., 2017), South Africa (Iweriebor et al., 2017), Israel (Thomson et al., 2018), Malaysia (Koh et al., 2018), Pakistan (Rehman et al., 2019), Corsica, France (Cicculli et al., 2019), China (Li et al., 2019) and America (Gajadhar et al., 2010; Cabezas-Cruz et al., 2012; Aguiar et al., 2014; Carvalho et al., 2016). Moreover, *E. minasensis* infects not only bovines (Gajadhar et al., 2010; Aguiar et al., 2014; Hailemariam et al., 2017) and cervids (Lobanov et al., 2012), as similar cases have also been reported in dogs (Thomson et al., 2018).

Molecular survey encompassing the genus *Ehrlichia* are usually carried out based on the disulfide bond formation protein gene (*dsb*), since this nucleic acid regions have conserved protein domains and are functionally conserved among bacteria, being excellent targets for genus-specific molecularly based assays. (McBride et al., 2002; Labruna et al., 2007; Aguiar et al., 2014; Aguiar et al., 2019a).

The purpose of this study was to determine the seroprevalence and molecular detection of *Ehrlichia* sp. in dairy cattle from Ji-Paraná municipality, in the state of Rondônia, and associate with possible risk factors for infection.

MATERIALS AND METHODS

Study site

This study was conducted in the municipality of Ji-Paraná (10º 52' 42" S, 61º 56' 41" W), located in the state of Rondônia, western Brazilian Amazon region, which is the third largest milk producer in the state. Crossbred dairy cows were sampled from September 2012 to November 2013. In addition, samples were obtained for an alternate study on the seroprevalence of *Neospora caninum* (Boas et al., 2015).

The sampling premises followed the six strata of milk production (rural sectors), in accordance with data from the Technical Assistance and Rural Extension Association of the State of Rondônia (Figure 1).

Sampling procedures

Blood and serum samples were collected in family farms, from dairy cows at the reproductive age (≥ 24 months old) and without clinical signs. Samples were selected in each stratum, considering two factors: farms and dairy cows. In determining the number of farms to be sampled, both the number of farms and the number of animals in each sector were taken into consideration. To select the stages of the main sample, a systematic draw was conducted among the farms in each sector, and another draw among the dairy cows.

The sample size was determined by the statistical formula $N_0 = [Np(1-p)/(N-1)(d/Z_{\alpha/2})^2+p(1-p)].deff$. The population size considered was 34,527 animals with prevalence estimated to be 50%, maximum error of 5%, 95% confidence interval, and design effect (*deff*) of 1.5. For blood sample analysis, the sample size of 570 animals was adjusted upward by 10% to account for the possibility of losses, to a total of 627 animals (approximately 10 animals for each farm, on 64 properties), with weighting according to the sampling design and taking into consideration the probabilities of selection among the farms and animals.

For serum analysis, the parameters were adjusted to a maximum error of 6% design effect of 1.2 and with a sample size of 318 animals (approximately five animals for each farm, on 64 properties). The complete sample design, considering the rural sectors of the Ji-Paraná municipality and the number of farms, is shown in Table 1.

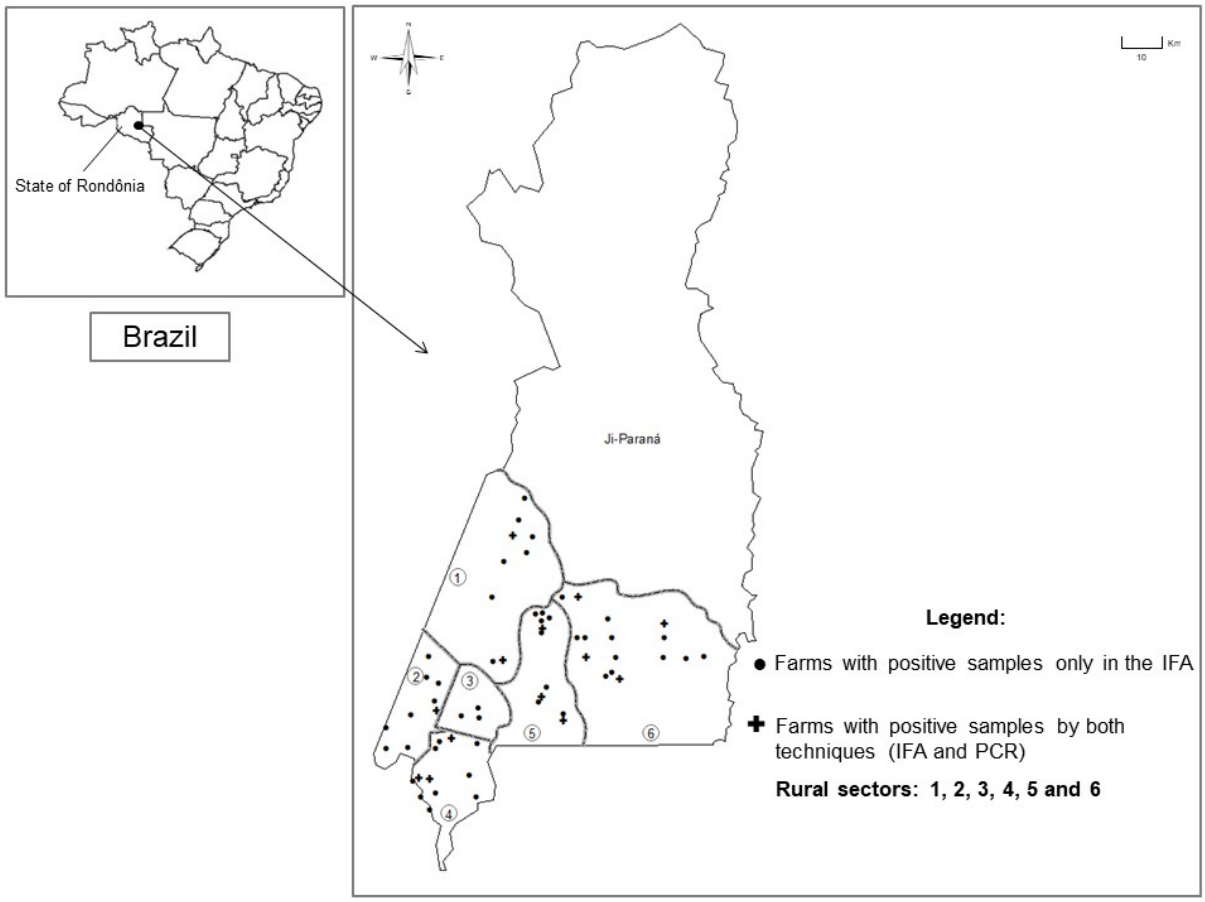

Figure 1 - Distribution of cow's samples that were positive in at least one technique used, indirect immunofluorescence assay (IFA) or polymerase chain reaction (PCR), which considered six strata of milk production (rural sectors), in accordance with data from the Technical Assistance and Rural Extension Association of the State of Rondônia (EMATER-RO), Ji-Paraná municipality, state of Rondônia.

Table 1 - Number of family farms, number of cows (≥ 24 months of age), number of farms sampled per rural sector, from September 2012 to November 2013, of the municipality of Ji-Paraná, Rondônia, Brazil.

Sector	Number of farms	Number of dairy cows	Number of farms sampled	Number of dairy cows sampled - blood	Number of dairy cows sampled – serum
1	122	5,246	9	75	45
2	124	5,952	9	85	45
3	54	2,268	4	40	20
4	176	8,096	13	130	65
5	143	4,433	11	108	55
6	237	8,532	18	172	90
Total	**856**	**34,527**	**64**	**610**	**320**

Samples and health questionnaire

Blood and serum samples were obtained from dairy cows on 64 farms. Blood and serum samples were collected from 10 and 5 dairy cows, respectively, from each farm (primary sampling unit). Overall, 610 blood (six farms did not have 10 cows for sampling) and 320 serum samples were collected (Table 1). Personnel at each farm were also given a questionnaire regarding general topics about the property to assess herd characteristics and management routines.

Detection of antibodies

Indirect immunofluorescence assay (IFA) was performed to detect antibodies against *Ehrlichia* sp. and these antibodies were evaluated using the Cuiabá 16 strain of *E. canis* with a cut-off point at an initial dilution of 1:40 (Aguiar et al., 2007). Briefly, the samples were diluted in PBS (pH 7.2) and applied to the IFA slides containing the antigen previously fixed using acetone. Negative and positive control sera were included in each slide. Anti-bovine IgG conjugated (Sigma Diagnostics, St. Louis, Mo, USA) was added at dilution 1:800. Glycerin (pH 8.5) was added to each slide to coverslip. Seroreactions were visualized under an epifluorescence microscope (Scope.A1 Zeiss) using a 40X objective. In each slide, a serum previously shown to be non-reactive (negative control) and a known reactive serum (positive control) were included.

Polymerase chain reaction (PCR) and sequencing

Blood sample was subjected to DNA extraction using phenol-chloroform mixture (Sambrook and Russel, 2011), and the obtained DNA was stored at –20 °C until molecular analyses. Heminested PCR was used to amplify a 401-bp amplicon of *dsb* gene (Dsb-330 5′-GATGATGTCTGAAGATATSAAACAAAT-3′ and Dsb-720 5′-CTATTTTACTTCTTAAAGTTGATAWATC-3′) in the first reaction and a 349-bp amplicon (Dsb-380 5′-ATTTTTAGRGATTTTCCAATACTTGG-3′ and Dsb-720) in the second reaction (Almeida et al., 2013). Amplicons were purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and were sequenced in an automatic sequencer (ABI DNA Model 3500 Series Genetic Analyzer), all in accordance with the manufacturers' protocols. The sequences obtained were subjected to BLAST analyses (Altschul et al., 1990) to determine closest similarities available in GenBank.

Statistical analysis

Statistical analyses were performed using the R statistical package (R Development Core Team, 2013), packages survey (Lumley, 2014), and spatial difference (spdep) (Bivand

et al., 2013). The associations between positivity for *Ehrlichia* sp. and the variables were analyzed using the chi-square (χ^2) test, with a significance of 5%. The covariate variables analyzed were as follows: livestock production systems; occurrence of abortion; number of cows aged 24 months or older; and presence of weak calves, when observed (Table 2).

Table 2 - Relationship between the presence of *Ehrlichia* sp. by the PCR method and IFA method and the variables analyzed by property.

Variables analyzed	Presence of *Ehrlichia* sp. by the PCR method		p-value	Presence of *Ehrlichia* sp. by the IFA method		p-value
No. of cows aged 24 months or greater	**Positive**	**Negative**		**Positive**	**Negative**	
<25	0	9	0.2689	8	1	0.0103*
25 a 50	3	16		18	1	
51 a 100	9	22		31	0	
>100	1	4		3	2	
Creation system	**Positive**	**Negative**		**Positive**	**Negative**	
Extensive	10	42	0.6360	48	4	0.9999
Semi-Extensive	3	7		10	0	
Semi-Intesive	0	2		2	0	
Occurrence of abortion	**Positive**	**Negative**		**Positive**	**Negative**	
Yes	6	30	0.6108	33	3	0.6252
No	7	21		27	1	
Presence of weak calves	**Positive**	**Negative**		**Positive**	**Negative**	
Yes	5	21	0.9999	25	1	0.6404
No	8	30		35	3	

*Statistical significance

RESULTS

Of 610 blood samples tested, 15 (2.45%; 95% CI: 1.04-3.86%) were positive for the *dsb* gene of *Ehrlichia* sp. Partial sequences of the *dsb* gene obtained from blood samples were identical to each other and showed 100% identical (295/295 bp) to the correspondence sequences of *E. minasensis* available in GenBank (MH500007, MT212419, JX629808, MT212413). Partial sequence of *E. minasensis* obtained in this study were deposited in GenBank under the accession number KT314243.

Anti-*Ehrlichia* sp. antibodies were detected in 178 cows (53.96%; 95% CI: 46.63–61.29%). Endpoint titers ranged from 40 to 5,120, and cattle in four properties were not positive

for the antibodies. Regarding the variables analyzed, IFA results identified that the number of cows aged 24 months or greater was statistically significant (p = 0.0103), and in these cows, herds of approximately 51-100 individuals were more likely to be infected with *Ehrlichia* sp., followed by those with 25-50, <25, and > 100 cows (Table 2).
Serum samples from cows collected on 13 farms were positive by both techniques (IFA and PCR), while samples from 47 farms were positive only in IFA. The distribution between each rural sector showing these positive farms is shown in Figure 1.

DISCUSSION

Previous study conducted in the State of Mato Grosso has reported the occurrence of *Ehrlichia* sp. in bovines (Aguiar et al., 2014). The study reported an infection rate of 9.5% (30/314) through molecular detection by PCR. Recently, Aguiar et al. (2019a) isolated the *E. minasensis* strain Cuiabá from Holstein calves and later reported the complete genome of *E. minasensis* isolate from animals in the state of Mato Grosso (Aguiar et al., 2019b).

The infection rate was lower in this study than the rate reported by Aguiar et al. (2014). In this case, several factors must be taken into account. The greater susceptibility to *Rhipicephalus* (*B.*) *microplus* tick infestation of dairy cattle is cited as a possible explanation for their higher rate of infection. Furthermore, family agricultural practices, in most cases, do not use pure dairy breeds and instead crosses several breeds, which may ultimately become more resistant. However, there is no available information regarding susceptibility of cattle breeds against this *Ehrlichia* genotype.

Regarding the possible vector, previous study has identified *E. minasensis* (UFMG-EV) in *R.* (*B.*) *microplus*, suggesting its potential for infection and transmission (Cabezas-Cruz et al., 2012). Moreover, *R.* (*B.*) *microplus* tick infection has also been reported in region of Mato Grosso State, confirming the occurrence of transstadial transmission of this agent with (Carvalho et al., 2016), *R.* (*B.*) *microplus* as the possible vector. In addition, *E. minasensis* has been identified in several other tick species other than *R.* (*B.*) *microplus*, such as *Rhipicephalus appendiculatus* (Iweriebor et al., 2017), *Hyalomma marginatum* (Cicculli et al., 2019), and *Hyalomma anatolicum* (Rehman et al., 2019). In this study, it was not possible to confirm the possible vector responsible for *Ehrlichia* transmission, despite the presence of *R.* (*B.*) *microplus* tick in the study area (Brito et al., 2006; Pereira et al., 2008).

Data collection regarding the presence of anti-*Ehrlichia* sp. antibodies is important because it indicates exposure of an animal to *ehrlichial* infection. However, the results obtained in this study should be interpreted with caution, as these tests may be hindered by a lack of specificity within or between the several species of *Ehrlichia*. Al-Adhami et al. (2011) conducted an evaluation on cattle that were naturally or experimentally infected with *Ehrlichia* sp. BOV2010 and detected a seropositive response of cattle to *Anaplasma marginale* by both cellular enzyme-linked immunosorbent assay (ELISA) and IFA methods. Blood smear examination and/or PCR was performed to confirm and rule out the presence of *Anaplasma* spp. from *Ehrlichia* spp. Thus, an evidence of serological cross-reactivity between *Ehrlichia* sp. BOV2010 and *A. marginale* can be tested using the commercially available cELISA kit for *Anaplasma* and IFA for *A. marginale* antigen–coated slides. Lobanov et al. (2012) have also reported on the occurrence of cross-reaction during antibodies testing of deer sera using the cELISA kit. Thus, according to Cabezas-Cruz et al. (2019), the serological diagnosis for *E. minasensis* can be challenging owing to the cross-reactivity of antibodies against the recombinant antigens.

Cattle in regions where *A. marginale* is endemic, such as in the state of Rondônia with *A. marginale* infection rate of 98.6% (Brito et al., 2010), are posed to high risk of exposure to other rickettsial pathogens that may induce antibodies cross-reactive with *A. marginale* proteins and may lead to false-positive serological test results. Since a seropositive animal indicates infection with *A. marginale*, *Ehrlichia* sp., or *Anaplasma centrale*, differentiation should be based on further testing, and in areas where both infections may coexist. Therefore, positive results of anti-*Ehrlichia* sp. antibodies derived from this assay should be interpreted with caution.

Analysis of the variables showed that among cows aged 24 months or older and those in a herd of 51 to 100 individuals were more likely to be infected with *Ehrlichia* sp.

High herd density, typically observed in dairy farms, increases the likelihood of microbial transmission among animals, including *R. (B.) microplus*. Intensive farming system can favor the transmission of ticks between animals, mainly dairy breeds as they are more susceptible (Brito et al., 2006; Pereira et al., 2008).

Currently, there is no available information regarding the susceptibility of cattle breed against this *Ehrlichia* genotype. In contrast, it is possible that the susceptibility of dairy cattle to *R.* (*B.*) *microplus* may play a significant role in the increased transmission of *Ehrlichia* sp. in these cattle (Aguiar et al., 2014). Thus, the pathogenic potential of *Ehrlichia* sp. should be assessed. Aguiar et al. (2014) successfully established the infection

through experimental inoculation in calves and identified clinical signs and initial morula in blood smears by 28th day of the experiment. Animals that were enrolled in this study were apparently healthy and without any clinical signs. The chronic nature of *E. minasensis* infection explains the high amount of antibodies and low detection of DNA, since the detection of rickettsial bacterium in blood samples is rather probable during acute infection, a fact that directly increases the sensitivity of a molecular detection.

CONCLUSION

This study reports on the prevalence of antibodies against *Ehrlichia* sp. as well as the presence of *E. minasensis* DNA genotype in dairy cattle in the northern region of Brazil. Although the animals did not show any clinical signs, the chronic character of the infection might lead to productivity decrease. Although we carefully analyzed the data regarding antibodies, we believe that this bacterium circulates in this region of Brazil as its DNA was detected in blood sample of cows in this region. Results obtained from this study provided evidence of natural infection caused by *E. minasensis* to cattle from Brazil's western Amazon region.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Ricardo Vilas Boas for their valuable help during field sampling of cattle and to the Technical Assistance and Rural Extension Association of the State of Rondônia (EMATER-RO) for technical support. D.M. Aguiar, L. Nakazato, R.C. Pacheco, and V. Dutra are in receipt of productivity scholarships from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

ETHICS COMMITTEE

The Bioethical Committee for Animal Research of the Federal University of Mato Grosso approved the present study, under the protocol n. 23108.015662/12-5.

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