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Original Article

High prevalence of *Anaplasma marginale* in the Crioula Lageana cattle

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Abstract

Introduction: Bovine anaplasmosis is caused by the bacterium *Anaplasma marginale;* its transmission occurs through vectors such as ticks. Crioula Lageana is a native cattle breed from the South of Brazil used for beef production, with excellent meat quality. There are no studies of the epidemiology of this disease in Crioula Lageana even though tick damage is known to be frequent.

Methodology: Blood samples were collected from 311 Crioula Lageana cattle and subjected to DNA extraction and polymerase chain reaction (PCR) using specific primers for the Major Surface Protein 5 (*msp5)* gene for the detection of the bovine anaplasmosis agent. The animals were classified according to the gender, the category and the presence or absence of ticks at the time of collection. The animal owners completed an epidemiological questionnaire to determine factors that might be associated with anaplasma infection.

Results: The prevalence of *A. marginale* was 79.9%. The following factors were found to be protective against infection: I) the breeding objectives (whether animals were destined for beef production and trade or solely for beef production), II) tick control rate; and III) pregnant and lactating cows and calves as the categories least affected by the hemoparasite. The main risk factor for hemoparasite acquisition was the use of organophosphates and avermectins as acaricides.

Conclusions: Crioula Lageana cattle are in a situation of enzootic stability, with a high prevalence of *A. marginale* infection. The factors associated with the infection were: I) breeding objectives, II) tick control rate, III) the acaricides used, and IV) the most tick-parasitized categories of cattle.

Key words: Native breed; health; anaplasmosis; *msp5*.

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Introduction

Anaplasmosis, caused by the agent *Anaplasma marginale,* is a widely distributed disease [1] that leads to huge economic losses due to high morbidity and mortality rates and the costs of controlling the disease [2]. The transmission of the hemoparasite depends on vectors such as the *Rhipicephalus microplus* tick, which is present in regions with tropical and subtropical climates [3]. In Santa Catarina State, located in the South of Brazil, Crioula Lageana is a native cattle breed very important for beef production in the region. Tick damage is frequent in this breed; the damange is mainly related to the transmission of pathogens, such *Anaplasma marginale*, *Babesia bovis* and *Babesia bigemina* [4].

The epidemiology of anaplasmosis varies according to the region under study, due to climatic conditions and the distribution of vectors in different locations [5], and according to factors related to racial characteristics such innate resistance to *Boophilus microplus*. The latter factors may or may not predispose animals to the development of clinical disease [6]. Animals of the Crioula Lageana breed have high-quality meat that is valuable; the breed possesses hardiness and adaptability. However, this breed has become nearly extinct; currently, conservation center properties are being used to maintain the breed group [7].

Knowledge of the mechanism of infection of Crioula Lageana cattle by *A. marginale* is an important step towards the knowledge of the health status of these herds and represents an investment in the further exploration of the genetic potential of the breed in breeding programs, considering its excellent production characteristics.

Animal health data are fundamental in meeting the requirements of commercialization of animal products inside and outside Brazil [8]. Epidemiological surveys based on molecular techniques provide more accurate and reliable data than those based on serology [9]. Enzootic stability is a term used in the epidemiology of tick and tick-borne diseases, related to host-tick-pathogen interactions, with a low incidence of clinical disease. It affects cattle production because when this condition is not present, outbreaks can occur [10]. In recent surveys, the Santa Catarina Plateau was designated as a region of enzootic instability for anaplasmosis [11,12], so studies in this field are necessary. Knowledge of the risk factors associated with infection is important in the development of strategies and methodologies for disease prevention and control in populations.

This study aimed to present the first survey of epidemiological data on *A. marginale* infection in the Crioula Lageana cattle breed using molecular techniques, and to identify the factors associated with agent acquisition at breeding and conservation properties of the breed in the state of Santa Catarina, Brazil.

Methodology

Determination of sample size

For the evaluation of the prevalence of *A. marginale* in the population of Crioula Lageana cattle, the following formulas were used according to the Organización Panamericana de la Salud (OPAS) [13]:

$$n_0 = \frac{1.96^2[p(1-p)]}{(d)^2}$$

where n_0 is the number of samples, p is the expected prevalence and d is the margin of error. Assuming an estimated prevalence of 50% for positive samples, a 95% confidence interval and a margin of error of 5%, the result for n_0 was 384 animals. However, because this is a finite population, we proceeded to the following calculation:

$$n = \frac{N\ x\ n_0}{N + n_0}$$

where N is the total number of animals in the population. For the Crioula Lageana breed, this number is 1500. From these calculations, the final number of 306 animals to be sampled was obtained.

Animals and sampling procedures

Blood samples from 311 Crioula Lageana cattle (males and females, young and adults, all registered by the Associação Brasileira de Criadores da Raça Crioula Lageana (Brazilian Crioula Lageana Breeders Association; ABCCL)) were collected in vacuum tubes containing 10% EDTA as an anticoagulant. The samples were obtained in the fall of 2016. The animals were located at *in situ* conservation centers in the cities of Lages, Ponte Alta, Painel and Curitibanos, which are located in the Santa Catarina Plateau, latitude 27º 48' 58" S and longitude 50º 19' 34" W. The cattle were classified into categories; samples were obtained from 32 bulls (males over two years old), 141 cows (females over two years old), 66 heifers (females between one and two years old) and 72 calves (males and females up to one year old). The absence of steers was because the samples originated from *in situ* conservation centers of the breed, so males that were not intended for breeding had been sold for fattening. For the analysis with regard to sex, the animals were separated into 62 males and 249 females, without distinction with respect to age.

Physical examination

Physical examination was used to verify the presence of clinical signs compatible with the clinical disease; heart rate (HR), respiratory rate (RR), ruminal movements (RM), rectal temperature and mucosal color were measured. All the variables measured were compared to the reference values for the specie (Data not shown).

DNA extraction

The blood samples collected from the animals were frozen at -20°C; after thawing, they were immediately subjected to DNA extraction using a commercial kit (ReliaPrep™ Blood gDNA Miniprep System – Promega – Madison, Winsconsin, EUA) according to the manufacturer's instructions. After extraction, the concentration of each DNA sample was measured in a spectrophotometer (NanoDrop 2000® Thermo Fischer – Waltham, Massachusetts, EUA), and the DNA was diluted with ultrapure water to a minimum concentration of 20 ng/μL.

Molecular analysis

To verify the presence of *A. marginale* in the animals, Major Surface Protein 5 (*msp5*) gene of *A. marginale* was chosen [9]. To amplify a 458-bp fragment of *msp5*, we used the following primers: (1) *msp5* F: 5'- CGC AGA TCT AGC AAA ATC GGC GAG AGG TTT ACC ACT TC -3' and (2) *msp5* R: 5'- GCG CTG CAG TGG CGC AAA ATG CCC GAC ATA CC -3') [9].

The PCR reaction was conducted in 0.2-mL microtubes in a final volume of 25 μL. The reaction

mixture contained 1 U Taq polymerase (Promega Corporation, Wisconsin, USA), 8.5 pmoles of each primer, 0.2 mM nucleotides (dNTPs), 3.5 mM magnesium chloride; PCR buffer at 1X final concentration, 3 µL of DNA (concentration between 20 and 100 ng/µL) and ultra-pure water to make up the final volume. Negative and positive controls containing *A. marginale* DNA (provided by Embrapa Amazônia Oriental, Belém do Pará, Brazil) were used for each reaction; the negative controls, which were used to guarantee the quality and specificity of the technique, contained the same components except that the genomic DNA was replaced with DNAse-free ultra-pure water. The temperature conditions applied in the thermal cycler included initial denaturation at 95ºC for two minutes, 30 cycles of 94ºC for 1 minute, 54.2ºC for 1 minute and 73ºC for 1 minute, and a final extension at 73ºC for 7 minutes.

Electrophoresis of the amplification products was conducted in a horizontal apparatus with 2% agarose gels containing dye. The first gel lane contained a molecular weight marker of 100 bp used as a standard to determine the size of the bands in the samples. The electrical source was applied at 140 V and 400 mA for 1 h, and the bands were visualized by exposure to ultraviolet light. The presence of a single band close to 458 bp in size was indicative of positivity for *A. marginale*.

To confirm that the fragments obtained in the electrophoresis originated from the *A. marginale msp5* gene, positive samples were randomly chosen and sequenced by Ludwig Biotec (Brazil) using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). The sequenced fragments were analyzed using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All the fragments had the same sequence and showed 98% identity with the sequence KP347554.1, corresponding to the *msp5* gene in Gene Bank (https://www.ncbi.nlm.nih.gov/genbank/).

Associated factors

To determine the factors associated with the presence of *A. marginale* infection, an epidemiological questionnaire containing questions related to the farm profile was completed by the owners of each site analyzed.

Statistical analysis

Univariate analysis using the chi-square test ($p \leq 0.05$) and odds ratio analysis were performed to compare the rates of infection by *A. marginale* in cattle of different sex and category and to determine whether or not infection was associated with the presence of ticks on the animals at the time of sampling. The statistical model applied to the questionnaire data was developed via univariate analysis using the chi-square test ($p \leq 0.05$). For the questions that yielded significant results in the first analysis, a multivariate analysis was conducted through logistic regression ($p \leq 0.05$). Questions that presented multicollinearity in the second analysis were excluded from the evaluation to enable the verification of the association between the presence or absence of the agent and the associated factors.

Ethics committee approval

The study was approved by the Comitê de Ética em Experimentação Animal (Committee of Ethics in Animal Experimentation; CETEA) of the Santa Catarina State University (UDESC) under protocol number 2461171115 and by the Comitê de Ética em Pesquisa com Seres Humanos (Committee for Ethics in Research with Humans; CEPSH) of the UDESC under protocol number CAAE 2.068.771.

Results

The prevalence of *A. marginale* infection in the sample was 79.7% (248/311). All animals positive for infection were considered subclinical, as no changes were detected on physical examination and all of the measured physical parameters remained within the reference values for the species (data not shown).

When the animals were evaluated for the presence or absence of infection by sex and category, it was found that of all the animals sampled, 249 (249/311, 80.1%) were female; of these, 199 (199/249; 79.9%) were positive for the agent. Of the 62 males (62/311, 19.9%) evaluated, 49 (49/62, 79.0%) presented positive results. There was no difference between the sexes in the percentage of infected cattle (p=0.983). Of the 32 bulls (32/311, 10.3%) evaluated, 26 (26/32, 81.2%) were positive; of the 66 heifers (66/311, 21.2%), 57 (57/66; 86.4%) were positive. Of the 141 cows (141/311, 45.3%), 115/141 (81.6%) showed positive PCR findings for *A. marginale*; similarly, of the 50 calves (50/72, 69.44%), 72/311 (23.15%) were positive for infection. The chi-square test did not show significant differences ($p = 0.077$) among these categories in the proportions of positive animals.

The relationship between the presence of ticks parasitizing the animals at the time of sampling and positivity for *A. marginale* infection was also evaluated. The analysis showed no relationship between these two variables in the chi-square test ($p = 0.274$).

In the analysis of the responses in the questionnaires completed by the owners, the variables associated with the presence of the infection were: I) breeding objective ($p < 0.001$), II) the contact of cattle with other animal species ($p < 0.001$), III) the replacement of animals ($p = 0.006$), IV) previous cases of anaplasmosis ($p = 0.003$), V) the period of greatest infestation by ticks ($p < 0.001$), VI) the presence of hematophagous insects ($p = 0.002$), VII) tick control rate ($p = 0.002$), VIII) the use of acaricides ($p < 0.001$), IX) the timing of treatment for ticks ($p < 0.001$), and X) the categories of animals with high tick infestation rates ($p < 0.001$) (Table 1).

Questions for which a value of $p \leq 0.05$ was obtained were considered significant in the univariate analysis and were thus subjected to multivariate analysis; the factors that presented multicollinearity

Table 1. Univariate analysis of the factors associated with A. marginale infection determined by the PCR technique for the Crioula Lageana cattle breed in Santa Catarina State, Brazil.

Variables	*A. marginale* infection				*p*
	Positive		Negative		
	N	%	N	%	
Number of animals on the property					
51 to 100	42	13.5	5	1.6	0.113
More than 100	206	66.2	58	18.7	
Breeding objectives					
Meat, reproduction and animal trade	134	43	31	10	
Meat	14	4.5	3	1	
Reproduction and animal trade	58	18.6	3	1	< 0.001
Meat and animal trade	14	4.5	24	7.7	
Sale	28	9	2	0.7	
Farm size					
50 to 100 ha	14	4.5	3	1	0.972
Greater than 100 ha	234	75.2	60	19.3	
Contact of cattle with other animal species					
Equine, dog and sheep	63	20.3	9	3	
Swine	28	9	2	0.6	
Equine and dog	14	4.5	24	7.7	< 0.001
Equine, swine, dog and cat	58	18.6	3	1	
Equine, dog, cat, bird and sheep	71	22.8	22	7	
Equine, dog, cat and wild animals	14	4.5	3	1	
Contact with cattle from other properties					
Yes	135	43.4	36	11.6	0.807
No	113	36.3	27	8.7	
Replacement of animals					
Own herd (No)	91	29.3	11	3.5	0.006
Own herd and other properties (Yes)	157	50.5	52	16.7	
Veterinary care					
Yes	105	33.8	35	11.2	0.082
No	143	46	28	9	
Previous cases of anaplasmosis					
Yes	77	24.8	33	10.6	0.003
No	171	55	30	9.6	
Period of greatest infestation by ticks					
Fall	72	23.1	6	2	
Summer	99	31.8	24	7.7	< 0.001
Fall and spring	14	4.5	24	7.7	
Summer and fall	63	20.2	9	3	
Presence of hematophagous insects					
Yes	190	61	60	19.4	0.002
No	58	18.6	3	1	
Do tick control?					
Yes	176	56.6	57	18.3	0.002
No	72	23.2	6	2	

Table 1 (continued). Univariate analysis of the factors associated with A. marginale infection determined by the PCR technique for the Crioula Lageana cattle breed in Santa Catarina State, Brazil.

Variables	*A. marginale* infection				*p*
	Positive		Negative		
	N	%	N	%	
Use of acaricides					
Pyrethroids	91	29.3	36	11.6	
Organophosphates and avermectins	99	31.8	24	7.7	< 0.001
Avermectins	58	18.6	3	1	
Time of treatment for ticks and anaplasmosis					
Fall	72	23.2	6	2	
Spring and fall	14	4.5	24	7.7	
Spring, summer and fall	71	22.8	22	7	< 0.001
Summer	28	9	2	0.6	
Summer and fall	63	20.2	9	3	
Most tick-parasitized categories					
Pregnant, lactating cow and calf	14	4.5	24	7.7	
Lactating cow	14	4.5	3	1	< 0.001
Calf	220	70.7	36	11.6	

were excluded from the model, as the real relationship between the variables was not determined.

Logistic regression was used to identify factors associated with anaplasmosis: the objective of meat production ($p = 0.017$), regular tick control ($p < 0.001$) and the groups of pregnant or lactating cows and calves ($p < 0.001$) were considered less susceptible to the acquisition of hemoparasites. The use of acaricides ($p = 0.004$) was a risk factor for *A. marginale* infection. (Table 2).

The multivariate analysis, which was conducted through binary logistic regression, aimed to verify whether the associated factors were in fact predictors of *A. marginale* infection.

Discussion

This is the first study of the prevalence of *A. marginale* in the Crioula Lageana cattle breed. The epidemiological data revealed that the farms evaluated are in a situation of enzootic stability according to Mahoney [14], with the presence of hemoparasite-infected ticks but with a low incidence of clinical disease in the cattle. All animals were asymptomatic at the time of sampling. Once the biological vectors are circulating in the animals and in the environment, immunity against *A. marginale* is maintained, keeping the animals free of clinical disease and reinforcing the importance of maintaining contact between the cattle with the vectors.

Situations of enzootic instability are uncommon in Brazil. In the Southern part of Brazil, regional differences in the epidemiology of infection may be due to the sampling times and the methods used to determine the prevalence; samples collected in the summer may reveal greater numbers of positive animals [15] due to the frequent infestation of the animals by the transmitting tick. In the regions in which sampling was conducted duringwinter and spring, the number of animals considered positive was low, and these regions were classified as being in a state of enzootic instability. They included the Center-South [16], the Northwest [17] and the Southwest of Paraná State [18]. All of these studies used serology to diagnose the infection. The use of tests such as ELISA, which detect antibodies against the agent and not its presence in the blood of the animals at the time of sampling, can lead to overestimation of infection prevalence in the herds [19].

In Santa Catarina State, a recent study classified the region of the Santa Catarina Plateau as being in a state of enzootic instability for anaplasmosis based on results obtained using the multiplex-PCR technique, which indicated a prevalence of 27.24% [12]; the data

Table 2. Multivariate analysis of the factors associated with A. marginale infection detected by PCR in the Crioula Lageana cattle breed, Santa Catarina State, Brazil.

Risk factor	P value	OR	95% CI	Coefficient	S.E.
Production purpose of husbandry	0.017	0.831	0.713 - 0.968	- 0.185	0.078
Tick control	< 0.001	0.066	0.017 - 0.250	- 2.724	0.682
Use of acaricides	0.004	2.114	1.262 - 3.542	0.749	0.263
Categories most parasitized by ticks	< 0.001	0.567	0.415 - 0.773	- 0.568	0.159

Significant association at the 5% level. OR = odds ratio; CI = 95% confidence interval; S.E. = standard error of estimate.

included the occurrence of an outbreak in the region in 2013 [11]. These data, however, were not confirmed in farms breeding the Crioula Lageana, where we measured a prevalence of 80%. This variation may be associated with the time at which sampling was conducted, since during the summer and fall, when blood samples were collected for this study, greater numbers of *Boophilus microplus* larvae and consequently more parasitized cattle are present [20], favoring *A. marginale* infection. The absence of regular control of the vectors and the extensive husbandry of the animals favor constant contact of the animals with the hemoparasite, reinforcing the animals' immunity against the causative agent of the clinical disease.

There was no relation between the sex of the animals and *A. marginale* infection ($p = 0.983$) [21,22]. Also the category of animal was not found to be a factor affecting the prevalence of infection in the Crioula Lageana. This is in agreement with the literature, which associates infection with the peripartum period in cows, as in this period, the concentration of immunoglobulins in females is reduced [23]. This state of immunosuppression, aggravated by hormonal changes, would favor the introduction of the parasite *A. marginale* [23]. M'Ghirbi *et al.* [24] found that the *A. marginale* infection rate was higher in young animals than in adults since the latter were exposed in higher rate to ticks, which are the agents of transmission. For the Crioula Lageana, such a difference was not verified ($p = 0.077$).

The presence of ticks on the animals at the time of sampling was not found to be associated with infection ($p = 0.274$). Abdela *et al.* [21] also found no association of ticks with *A. marginale* infection and hypothesized that other sucking arthropods and fomites may play an important role in its transmission. In areas of Central America and Africa where the tick vector is absent, mechanical transmission via fomites is considered the primary means of transmission of bovine anaplasmosis [25].

Among the factors associated with infection, the breeding objectives ($p < 0.017$) were associated with reduced rates of infection by *A. marginale*. The properties that had, among other purposes, the objective of animal reproduction, were associated with the highest rates of inoculation. These data may be associated with changes caused by cows' gestation and lactation. Nevertheless, the breeding objectives do not increase the risk of infection by *A. marginale* and may even contribute to controlling the disease, especially on properties that work exclusively with animals destined for the production of meat and for animal trade.

Likewise, tick control was identified ($p < 0.001$) as a negative risk factor for acquisition of infection. Of the 311 animals evaluated, 56.6% received regular tick control, and these animals were slightly less likely (OR = 0.066) to acquire the infection. Wesonga *et al.* [22] found no relationship between regular tick control and *A. marginale* infection in a multivariate analysis, and attributed this finding to the absence of real efficacy of the active principles used for control.

The classes of active ingredients used in tick control were also associated with the presence or absence of *A. marginale* infection ($p = 0.004$). A study of the resistance of ticks to acaricides in Mexico showed that these arthropods are resistant to more than one class of acaricide [26]. In the cattle population investigated in our study, the class of acaricide used or the use of a combination of different classes of acaricides were associated with an increase of more than two-fold in the likelihood of the animals being positive for the anaplasmosis agent. In farms that use a combination of organophosphates and avermectins (OR = 2.114), there are indications that ticks may be resistant to several classes of commonly used acaricides. The lack of regularity in the application of acaricides on most of the properties can lead to resistance of the ectoparasites to the active ingredients used, favoring the presence of ticks parasitizing the cattle and thus maintaining *A. marginale* infection rates.

According to information obtained from the owners of the animals, the category of animal most infested by ticks was calves. Animal category is a significant factor ($p < 0.001$) with respect to tick infestation and positivity for *A. marginale*. The effect of age can also be confused with the animals' physiological state. When age is evaluated in studies of bovine resistance to ticks, older animals tend to show more susceptibility to infestation than younger ones [27,28]. This was not confirmed in this work, according to the observations made by the owners. Thus, observations made over an extended period, with tick counts conducted at different times, are necessary to confirm the behavior of the ectoparasites in each animal category, given that the data we obtained are not consistent with the data on infestations that have been reported in the literature.

Most of the factors that were found to be significant in the multivariate analysis were associated with odds ratio values that represented a reduction in the chances of infection (OR < 1). These data may reveal factors that can be considered positive in the management of cattle of this breed. The protective factors, according to the OR, can reduce the likelihood of infection by *A. marginale* without, however, eliminating the parasite,

thus maintaining a situation of enzootic stability in the population.

Conclusions

A high prevalence of *A. marginale* infection in the Crioula Lageana cattle breed was revealed by the use of the PCR technique to identify infected animals. Regarding the factors associated with *A. marginale* infection, animals whose production purpose was for sale and/or meat production, those who received regular tick control, and specific categories of animals parasitized by ticks, such as pregnant, lactating cows and calves, were considered less susceptible to the acquisition of hemoparasites. The use of organophosphates in combination with avermectins as acaricides were a risk factor for *A. marginale* infection, probably due the acaricide resistance. We recommend further studies in order to establish a more accurate picture of how these factors are associated with the rates of infection of *A. marginale*.

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Authors' contributions

MSC, JHF, ALFL, CIGV and LCM designed the study; MSC, JMV and KMS performed the experiment; MSC, JHF, CIGV and LCM analyzed the data and wrote the manuscript.

References

1. Kocan KM, de la Fuente J, Blouin EF, Coetzee JF, Ewing SA (2010) The natural history of Anaplasma marginale. Vet Parasitol 167: 95-107.
2. Brown CGD (1997) Dynamics and impact of tick-borne diseases of cattle. Trop Anim Health Prod 29: 1S-3S.
3. Guglielmone AA (1995) Epidemiology of babesiosis and anaplasmosis in South and Central America. Vet Parasitol 57: 109-119.
4. Paim F, Souza APD, Bellato V, Sartor AA (2011) Selective control of Rhipicephalus (Boophilus) microplus in fipronil-treated cattle raised on natural pastures in Lages, State of Santa Catarina, Brazil. Rev Bras Parasitol Vet 20: 13-16.
5. Sharma A, Das Singla L, Tuli A, Kaur P, Bal MS (2015) Detection and assessment of risk factors associated with natural concurrent infection of *Trypanosoma evansi* and *Anaplasma marginale* in dairy animals by duplex PCR in eastern Punjab. Trop Anim Health Prod 47: 251-257.
6. Bock, RE, De Vos, AJ, Kingston, TG, & McLellan, DJ (1997). Effect of breed of cattle on innate resistance to infection with *Babesia bovis*, *B bigemina* and *Anaplasma marginale*. Aust Vet J, 75: 337-340.
7. Martins VMV, Veiga TF, Martins E, Quadros SAF, Cardoso CP, Ribeiro JAR (2009).Lagean Creole breed: the mainstay of yesterday, the work of today and the opportunity of tomorrow . Lages: ABCCL. [Book in Portuguese].
8. Juliano RS, Fioravanti MCS, Brito WMEDD, Abreu UGPD, Souza SND (2014)Seroepidemiology of bovine leukemia (BLV) in curraleiro cattle in the states of Goiás and Tocantins, Brazil . Ciência Animal Brasileira 15: 289-295. [Article in Portuguese].
9. Brito LG, Oliveira MCDS, Rocha RB, Netto FGDS, Marim AD, Souza GCRD, Vendrame FB, Moura MMDF (2010) *Anaplasma marginale* infection in cattle from south-western Amazonia. Pesqui Agropecu Bras 30: 249-254.
10. Jonsson, NN, Bock, RE, Jorgensen, WK, Morton, JM, Stear, MJ (2012) Is endemic stability of tick-borne disease in cattle a useful concept? Trends parasitol, 28: 85-89.
11. Canever MF, Vieira LL, Reck C, Richter L, Miletti LC (2014) First evaluation of an outbreak of bovine babesiosis and anaplasmosis in Southern Brazil using multiplex PCR. Korean J Parasitol 52: 507-511.
12. Vieira, LL, Canever, M.F, Cardozo, LL, Cardoso, CP, Herkenhoff, ME, Neto, AT, Miletti, LC (2019). Prevalence of Anaplasma marginale, Babesia bovis, and Babesia bigemina in cattle in the Campos de Lages region, Santa Catarina state, Brazil, estimated by multiplex-PCR. Parasite epidemiol control, 6, e00114: 1-8.
13. OPAS (Organización Panamericana de la Salud) (1979).Biostatistics: procedures for prevalence studies by researchers . Buenos Aires: Organización Panamericana de la Salud. [Book in Spanish]
14. Mahoney DF (1975). The diagnosis of babesiosis in Australia. In Wells EA, editor. Workshop on hemoparasites (*Anaplasmosis* and *Babesiosis*). Colombia: CIAT. 49-62.
15. Andrade G, Vidotto O, Vidotto M, Yoshihara E, Kano F, Amaral C (2001) Seroprevalence of *Anaplasma marginale* in dairy cattle and, studies on the dynamics of natural infection of Holstein calves in Southern Brazil. Semin Cienc Agrar 22: 155-159.
16. Marana ERM, Dias JA, Freire RL, Vicentini JC, Vidotto MC, Vidotto O (2009) Seroprevalence of marginale in cattle from the Center-South region of the state of Paraná, Brazil, by a competitive enzyme immunoassay using recombinant protein MSP5-PR1. Rev Bras Parasitol Vet 18: 20-26. [Article in Portuguese].
17. Yoshihara E, Vidotto O, Yamamura MH, Marana E, Pacheco R, Silveira A (2003) Studies of natural infection with *Anaplasma marginale* in nelore cattle in the Umuarama municipality, Parana State, Brazil. Rev Bras Parasitol Vet 12: 21-26.
18. Sott T, Franciscato C, Neto AFDS, Nascif Júnior IA, Freitas FLDC (2016) Enzootic instability for bovine anaplasmosis on family farms located in southwestern Paraná, Brazil. Rev Bras Parasitol Vet 25: 497-500.
19. Wagner G, Cruz D, Holman P, Waghela S, Perrone J, Shompole S, Rurangirwa F (1992) Non-immunologic methods

of diagnosis of babesiosis. Memórias do Instituto Oswaldo Cruz 87: 193-199.
20. de Souza AP, Gonzales JC, Ramos CI, Paloschi CG, de Moraes AN (1988)Boophilus microplus seasonal variation in the Plateau of Santa Catarina . Pesqui Agropecu Bras 23: 627-630. [Article in portuguese].
21. Abdela N, Ibrahim N, Begna F (2018) Prevalence, risk factors and vectors identification of bovine anaplasmosis and babesiosis in and around Jimma town, Southwestern Ethiopia. Acta Trop 177: 9-18.
22. Wesonga FD, Gachohi JM, Kitala PM, Gathuma JM, Njenga MJ (2017) Seroprevalence of *Anaplasma marginale* and *Babesia bigemina* infections and associated risk factors in Machakos County, Kenya. Trop Anim Health Prod 49: 265-272.
23. da Silva JB, da Fonseca AH (2014) Risk factors for anaplasmosis in dairy cows during the peripartum. Trop Anim Health Prod 46: 461-465.
24. M'Ghirbi Y, Bèji M, Oporto B, Khrouf F, Hurtado A, Bouattour A (2016) *Anaplasma marginale* and *A. phagocytophilum* in cattle in Tunisia. Parasit Vectors 9: 556.
25. Figueroa JV, Alvarez JA, Ramos JA, Rojas EE, Santiago C, Mosqueda JJ, Vega CA, Buening GM (1998) Bovine babesiosis and anaplasmosis follow-up on cattle relocated in an endemic area for hemoparasitic diseases. Ann N Y Acad Sci 849: 1-10.
26. Foil LD, Coleman P, Eisler M, Fragoso-Sanchez H, Garcia-Vazquez Z, Guerrero FD, Jonsson NN, Langstaff IG, Li AY, Machila N, Miller RJ, Morton J, Pruett JH, Torr S (2004) Factors that influence the prevalence of acaricide resistance and tick-borne diseases. Vet Parasitol 125: 163-181.
27. da Silva AM, Alencar MMD, Regitano LCDA, Oliveira MCDS (2010) Natural infestation of female beef cattle by ectoparasites in southeastern Brazil . Revista Brasileira de Zootecnia 39: 1477-1482. [Article in Portuguese].
28. Fraga AB, Alencar MMD, Figueiredo LAD, Razook AG, Cyrillo JNDSG (2003)Analysis of genetic and environmental factors that affect the infestation of ticks (Boophilus microplus) in bovine females of the Caracu breed. Revista Brasileira de Zootecnia 32: 1578-1586. [Article in Portuguese].

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