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PARASITOLOGY

Natural infection by *Anaplasma marginale* during the first weeks of life of calves on a dairy farm in the eastern Amazon

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ABSTRACT: This study detected *Anaplasma marginale* in calvesusing blood smears and nested PCR (nPCR) and to compare the results with the clinical signs presented by calves on a dairy farm in the municipality of Castanhal, located northeast of the state Pará (1°07'19.1"S and 47°53'53.0"W), eastern Amazon. To this end, 192 blood samples were collected from 24 animals at 1–20, 21–41 and 42–60 days of age. Blood smears and nPCR with primers for the *msp5* gene were performed. The prevalence of *A. marginale* was 61.5% (118/192) for the blood smear technique and nPCR (*msp5*). The manifestation of clinical signs of anaplasmosis also increased significantly over the course of the study ($P < 0.0001$), being lower in animals aged 1–20 days, but increasing among those aged 21–41 and 42–60 days. These signs were characterized by apathy, fever, weight loss, diarrhea, dehydration, and hypochromic mucous membranes. Regarding the evaluation of the diagnostic techniques, no significant difference was observed in the detection of *A. marginale* between the blood smear and nPCR ($P = 0.995$), but the agent's rickets increased on Day 47 ($P < 0.01$) in both tests, thereby demonstrating a near-linear pattern of increase in rickets over the 60 days, with a consequent decrease in globular volume. This shows that of the 24 animals studied, 21 were infected at some point during the study period. Additionally, there was no significant difference between blood smears and nPCR, probably due to medium and high parasitemia, which were directly related to the clinical signs and decrease in globular volume.
Key words: hemoparasite anemia, msp5, ricketsemia.

Infecção natural por *Anaplasma marginale* durante as primeiras semanas de vida de bezerros de uma propriedade leiteira da Amazônia Oriental

RESUMO: Objetivou-se, na construção deste trabalho, detectar *Anaplasma marginale* por meio do esfregaço sanguíneo e Nested PCR (nPCR) e comparar os resultados com os sinais clínicos apresentados pelos bezerros em uma propriedade leiteira localizada no município de Castanhal, região nordeste do estado do Pará (1°07'19,1"S e 47°53'53,0"W), Amazônia Oriental. Para isso, foram coletadas 192 amostras sanguíneas de 24 animais, divididos em três períodos: 1-20, 21-41 e 42-60 dias de idade. Foram realizados esfregaços sanguíneos e nPCR com iniciadores para o gene *msp5*. A prevalência de *A. marginale* foi de 61,46% (118/192) tanto para a técnica de esfregaço sanguíneo quanto para nPCR (*msp5*). A manifestação de sinais clínicos da anaplasmose também foi significativamente crescente ao longo do estudo ($P < 0,0001$), sendo menor em animais de 1 a 20 dias, mas expandindo-se entre os de 21 a 41 dias e 42 a 60 dias, esses sinais foram caracterizados por apatia, febre, perda de peso, diarreia, desidratação e mucosas hipocoradas. Quanto a avaliação das técnicas diagnósticas, não houve diferença significativa entre a detecção de *A. marginale* no esfregaço sanguíneo e na nPCR ($P = 0,995$), porém se observou aumento riquetsêmico do agente no 47º dia ($P < 0,01$) em ambos os testes, demonstrando, assim, um padrão de aumento da riquetsemia próximo ao linear ao longo dos 60 dias, com consequente diminuição do volume globular. Assim, demonstra-se que dos 24 animais estudados, 21 se infectaram em algum momento do período estudado, e não houve diferença significativa entre esfregaço sanguíneo e nPCR, em virtude, provavelmente, das parasitemias médias e altas, as quais estiveram diretamente relacionadas com os sinais clínicos e a diminuição do volume globular.
Palavras-chave: anemia hemoparasita, msp5, riquetsemia.

INTRODUCTION

Anaplasmosis is a common disease afflicting cattle in Brazil. The causative agent of the clinical manifestation is the rickettsia *Anaplasma marginale* (KOCAN et al., 2010), which can be transmitted biologically by ticks; mechanically by hematophagous arthropods (such as flies and mosquitoes) and contaminated fomites; and transplacentally (SILVA & FONSECA, 2014).

Anaplasmosis is endemic in tropical and subtropical zones (KOCAN et al., 2010; FERNANDES et al., 2019). BRITO et al. (2007) determined the prevalence rate of *A. marginale* in cattle herds in Rondônia and Acre to be 98.6% (1650/1627) and 92.86% (208/225), respectively. In

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the North and Center-West regions of Brazil, SILVA et al. (2015) verified the occurrence of antibodies against *A. Marginale*; the authors highlighted the Pará with a prevalence rate of 74.52% (506/679) for the rickettsia, determining it to be a region of enzootic stability.

Diagnosis using blood smears has shown insufficient sensitivity in detecting parasites in cattle considered to be healthy carriers. Therefore, molecular techniques have been used to detect hemoparasites in cattle populations, facilitating rapid determination of the risk of outbreaks (MOSQUEDA et al., 2012); these diagnostic methods have high levels of sensitivity and specificity. Blood smears are a routine method for diagnosing hemoparasites, especially when parasitemia is medium to high, but in animals with chronic infection, the method does not have sufficient sensitivity for parasite detection (BRITO et al., 2007).

Polymerase Chain Reaction (PCR) is a technique for amplifying specific sequences of deoxyribonucleic acid (DNA), which is highly sensitive in detecting small amounts of DNA in tissue or fluid samples (THEILER, 1910). Various modifications of PCR, such as the use of two consecutive PCRs including a second pair of internal primers, can increase the sensitivity and specificity of the original technique, thereby making the reaction more efficient. This technique is known as nested PCR (nPCR) (MACHADO et al., 1997).

TORIONI DE ECHAIDE et al. (1998) standardized nPCR for amplifying the *msp5* gene of *A. marginale*. In Brazil, studies frequently use PCR for the diagnosis of *A. marginale,* both in research on the genetic diversity of the agent (BAÊTA et al. 2015) and epidemiological studies (GONÇALVES et al., 2011; MOSQUEDA et al., 2012; ROMERO-SALAS et al., 2016). Recently, in Pará, a study was conducted on the genetic diversity of calves naturally infected with *A. marginale* (MONTEIRO et al*.,* 2023). However, studies using two diagnostic techniques and associating the results with the clinical signs presented by the animals have not been found in the region yet.

This study detected *A. marginale* using blood smears and nPCR, and to compare the results with the clinical signs presented by calves in the first 60 days of life on a dairy farm located in the municipality of Castanhal, in the northeastern region of Pará, eastern Amazon, to determine the period of greatest vulnerability of the animal to infection.

MATERIALS AND METHODS

Study area and animals

This study was conducted on a dairy farm located 21 km from Castanhal (1°07'19.1"S and 47°53'53.0"W). This area has an average annual temperature of 26 °C, with a maximum of 35 °C, and average annual rainfall between 2,500 mm and 3,000 mm, with a history of mortality from bovine anaplasmosis.

The selection of the calves was coordinated with the fixed-time artificial insemination (FTAI) protocol established on the property, which facilitated the collection schedules. The animals were born between April and May 2021. Thus, 24 mixed-breed calves of the Gir and Holstein breeds were selected and monitored from the first day of birth until 60 days of age. The clinical examination of the animals was performed according to DIRKSEN et al. (1993) within 24 hours of birth and subsequently on 20, 26, 34, 41st, 47, 54, and 60 days after birth (DPN). Each animal evaluated in the experiment had an anamnesis form with identification data and was examined on the same collection days as those set out above.

The animals were born in the maternity paddock and ingested colostrum in the first six hours of life; they stayed with their mothers for up to 20 days and were then placed in collective calf pens with animals from other age groups (from one month to one year old) with access to mineral salt, feed, and water at will. During this period, they were also released onto pasture (*Panicum maximum* cv. Mombaça) in the morning and afternoon in the company of their mother.

Sample collection and processing

The first blood samples were collected up to 24 hours after birth, and at 20, 26, 34, 41st, 47, 54, and 60 DPN, totaling 192 blood samples from the 24 experimental animals.

Samples were collected via jugular venipuncture in sterile vacuum tubes with the anticoagulant Ethylenediamine Tetraacetic Acid (K_3 EDTA) for blood smears and globular volume, and an aliquot of the blood stored in *eppendorf* tubes (–20 °C) was sent for subsequent DNA extraction and PCR.

Blood smear, ricketsemia, and globular volume

Blood smears were made from whole blood fixed in methanol (Synth®) and stained with Giemsa (Sigma-Aldrich®). The positivity of the sample was determined by examining it under a light microscope (Olympus BX40) for the presence of intra-erythrocytic *A. marginale* inclusion corpuscles based on morphology (TORIONI et al., 1998; THEILER, 1910) or another hemoparasite.

The levels of rickettsia in naturally infected calves were determined by identifying infected cells in 40 randomly selected fields with

an estimate of 250–300 blood cells per field under a microscope, and the number of cells with rickettsia inside was calculated as a percentage. The animal was considered to be infected with *A. marginale* when it showed rickettsemia ≥ 0.01% (REINBOLD et al., 2010). The globular volume was determined according to HARVEY (2012).

DNA extraction and nested polymerase chain reaction (nPCR)

Blood DNA was extracted using the commercial Wizard Genomic DNA Purification Kit (Promega®) following the manufacturer's protocol. The total DNA extracted was used for the detection of *A. marginale* DNA through nPCR, using the primer oligonucleotides (GenBank accession M93392) Amar *msp5* eF (5'-GCATAGCCTCCGCGTCTTTC-3'),Amar*msp5*iF (5'-TACACGTGCCCTACCGAGTTA-3'), and Amar *msp5* eR (5'-TCCTCGCCTTGGCCCTCAGA-3'), described by SINGH et al. (2012), which produced 345 base pairs.

The conditions used for amplification in a thermal cycler (Bio Rad T100TM) for the first and second reactions of *msp5* were as follows: initial denaturation at 94 °C for two minutes, followed by 35 cycles at 94 °C for one minute, 58 °C for one minute, and 72 °C for one minute, and a final extension at 72 °C for five minutes. The nPCR products were analyzed on a 1.5% agarose gel, stained with ethidium bromide. The reactions had a positive and negative control (ultrapure water).

Statistical analysis

The aim of the simple linear regression analysis was to evaluate the dynamics of *A. Marginale* ricketsemia over Days 1, 20, 26, 34, 41, 47, 54, and 60, corresponding to the collection days for all the animals. The graphs with the residuals and slope coefficients of the regression lines were obtained using Microsoft Excel 2019 software® (version 16.0).

The calves were divided into the following three development periods for better monitoring: 1–20, 21–41, and 42–60 days.

The Kolmogorov–Smirnov test was used to assess the normality of the quantitative data. ANOVA was used to assess whether the values for globular volume, smear diagnostic method, nPCR, and ricketsemia varied significantly between the days of analysis. Tukey's post-test was used when necessary.

The Chi-square test was used to check whether the detection of *A. marginale* by blood smear and the manifestation of clinical signs varied according to the days analyzed. All the statistical analyses were performed using the Bioestat 5.3 program, adopting a significance level of $\alpha = 0.05$.

RESULTS

Anaplasma marginale was detected in blood smears from calves in the presence and absence of clinical signs at some point during the study period. The clinical signs observed were as follows: apathy, fever, weight loss, diarrhea, dehydration, and hypochromic mucous membranes. Two of these animals had mixed infection with *Babesia bigemina* in blood smears, and only one merozoite of the agent was found in every two slides of the animal. Due to the low prevalence of *Babesia* sp., nPCR was performed only on the *A. marginale* positive animals.

Ticks were observed on the animals throughout the experiment. According to the owner, tick control was conducted with acaracides of different pharmacological bases, as advised by the farm's veterinarian.

The prevalence of positives for *A. marginale* inclusion corpuscles, detected using blood smear, was 61.46% (118/192 samples). Clinical cases of this disease were predominant between 42 and 60 days of age.

Table 1 shows the positivity for *A. marginale* in blood smears and the average Globular Volume according to the evaluation periods of the experimental animals. Significant decreases were observed in mean GV over the days ($P < 0.01$), which was higher in the animals assessed between 1 and 20 days and lower in those assessed between 42 and 60 days.

Figure 1 shows the temporal evolution of rickettsemia and the percentage of animals showing clinical signs according to the days of assessment. In this scenario, in the first 20 days, 8.3% and 16.7% (4/24) of the animals showed clinical signs; in the following 20 days (21–41 days), 79.14% of the animals showed rickettsia in a blood smear, with varying levels of globular volume. For example, 25% (6/24), 33.3% (8/24), and 58% of the animals showed clinical signs on Day 26, 34, and 41, respectively. In the last group (42–60 days), 95.83% of the animals were infected by the rickettsia, detected via blood smear and 46% (11/24), 67% (16/24), and 45.8% (11/24) of the animals showed clinical signs on Day 47, 54, and 60, respectively (Figure 1). The clinical signs included apathy, fever, hypochlorous mucous membranes, diarrhea, and dehydration. The detection of *A. marginale* using blood smears was

Table 1 - Positivity for *Anaplasma marginale* in blood smears and average globular volume (VG) according to each evaluation period of dairy calves from a property in the eastern Amazon.

Evaluation period[1]	----------Positivity among animals----------	----------Average VG[*] (min - max) ----------
1–20 days	8.3% (2/24)	30%[a] (15–48)
21–41 days	79.2% (19/24)	23%[b] (10–35)
42–60 days	95.8% (23/24)	19%[c] (9–32)

[1]Monitored weekly for 60 days.
[*]Different letters in the same column represent a statistically significant difference using ANOVA and Tukey's post-test ($P < 0.05$). Values with the same letters do not differ significantly. Min= minimum percentage; max= maximum percentage.

significantly higher in animals aged 21–41 and 42–60 days ($P < 0.0001$) (Table 1), and the manifestation of specific clinical signs was also significantly higher throughout the study ($P < 0.0001$), the signs being lower in animals aged between 1 and 20 days, but increasing in those assessed between 21 and 41 and 42 and 60 days.

In this study, a significant increase was observed in ricketsemia over the period the animals were monitored (Table 2, Figure 2). In the graph showing the dynamics of ricketsemia in the animals (Figure 2), the distribution of points close to the regression line was noted, with a value of $R^2 = 0.8828$, which shows a pattern of increasing ricketsemia close to linear, especially between Days 20 and 47. Notably, the peak of parasitemia occurred on Day 47, and until Day 60, such that ricketsemia maintained a pattern close to stability (plateau).

In this study, no significant differencewas observed between the detection of *A. marginale* inblood smears and nPCR ($P = 0.995$). Conversely, a significant increase was observed in the diagnosis of the agent up to Day 47 ($P < 0.01$), by blood smear and nPCR (Table 2). Importantly, 87.5% (21/24) of the animals showed clinical signs of anaplasmosis at some point during the study.

DISCUSSION

The northern region of Brazil has the ideal climate and temperature conditions for the maintenance and development of the *Rhipicephalus*

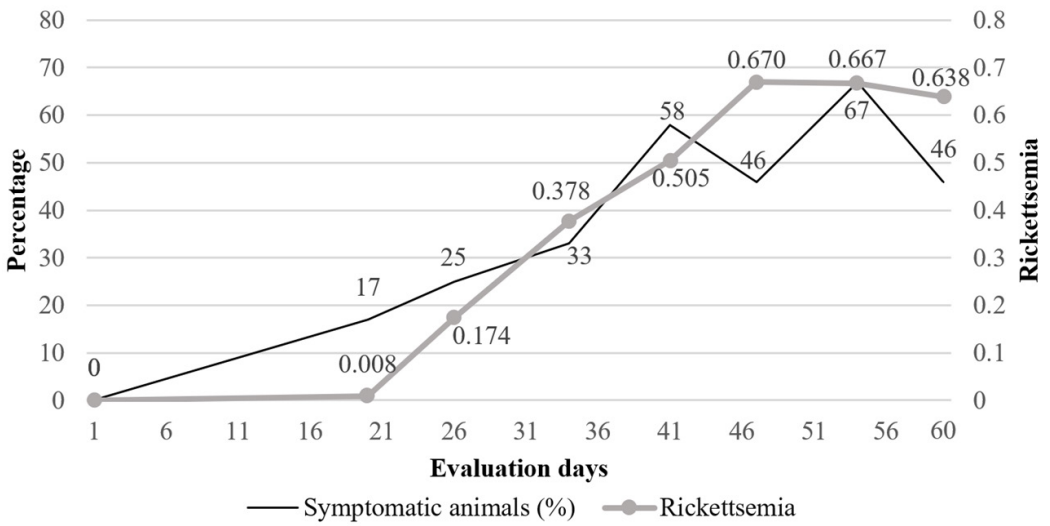

Figure 1 - Comparison between the temporal evolution of ricketsemia (bars) and percentage of calves showing the clinical signs of anaplasmosis, according to the days of treatment.
Evaluation (D1 to D60) on a dairy farm in the eastern Amazon.

Table 2 - Frequency of *Anaplasma marginale* established between blood smear and nPCR showing the percentage for each day of collection and mean rickets (number of infected cells / 40 random fields on the slide × 100) in calves evaluated on a dairy farm in the eastern Amazon.

Age/days	-------Average rickettsemia*-------	----------Frequency of positives (%)**----------	
		Blood smear	nPCR
1	0^{a}	0^{a}	0^{a}
20	$0.1^{a.b}$	$8.3\ (2/24)^{b}$	$12.5\ (3/24)^{b}$
26	$0.3^{a.b.c}$	$37.5\ (9/24)^{c}$	$37.5\ (9/24)^{c}$
34	$0.5^{b.e}$	$75\ (18/24)^{d}$	$70.8\ (17/24)^{d}$
41	$0.6^{c.e}$	$83.3\ (20/24)^{e}$	$83.3\ (20/24)^{e}$
47	$0.7^{d.e}$	$95.8\ (23/24)^{f}$	$95.8\ (23/24)^{f}$
54	0.7^{e}	$95.8\ (23/24)^{f}$	$95.8\ (23/24)^{f}$
60	0.7^{e}	$95.8\ (23/24)^{f}$	$95.8\ (23/24)^{f}$

*Different letters in the same column represent a statistically significant difference using ANOVA and Tukey's post-test ($P < 0.01$). Values with the same letters do not differ significantly.
**Different letters in the same row or column represent a statistically significant difference using the ANOVA test and Tukey's post-test ($P < 0.05$). Values with the same letters do not differ significantly.

microplus tick, which is the vector of *Anaplasma marginale*. BARROS-BATTESTI et al. (2006) stated that this tick can complete up to five generations per year, depending on the humidity and temperature conditions.

The constant presence of the biological vector *R. microplus*,intensive use of acaricides and chemoprophylaxis measures, persistence of the infection in the "bovine" reservoir, mechanical vectors (hematophagous flies), and contaminated fomites can contribute to an increase in the frequency of *A. marginale* in the herd (JAIMES-DUEÑES et al., 2017; REINBOLD et al., 2010). On the property studied, there was a constant presence of ticks parasitizing the experimental and other animals in the herd, which was the likely source of transmission,

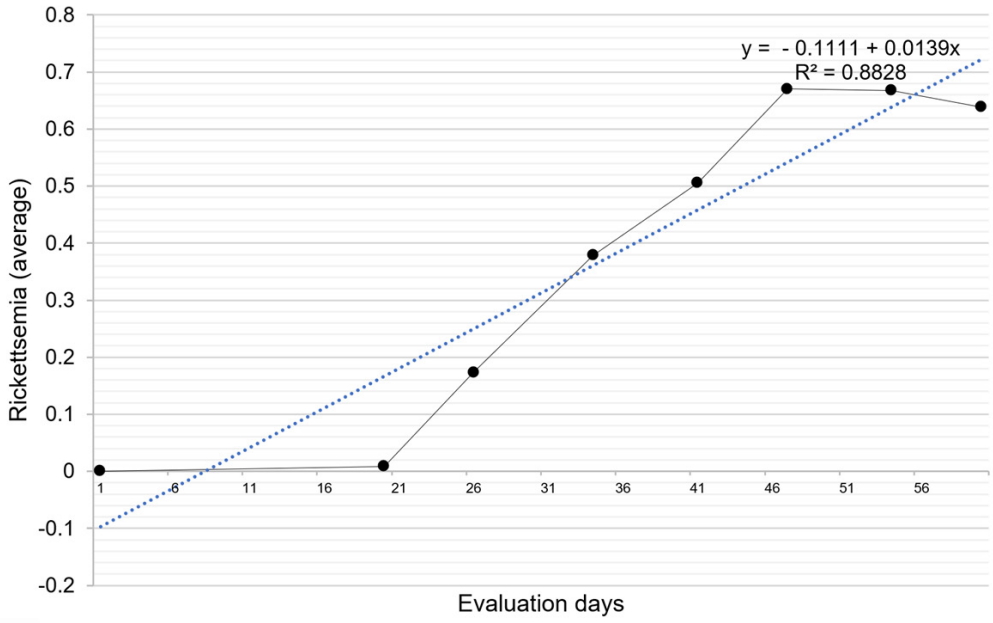

Figure 2 - Dynamics of ricketsemia averages over the study evaluation period (Days 1, 20, 26, 34, 41, 47, 54 and 60), with $R^2 = 0.8828$, in calves from dairy farms in the eastern Amazon.

given that transmission via fomites, such as needles, was unlikely due to the management adopted on the farm. Another factor that may have contributed to the maintenance of reservoir animals in the herd, or even the presence of different strains of *A. marginale,* was the fact that the property frequently introduced new cows acquired from different regions of Pará.

The study indicated that the period of greatest infection and presentation of clinical signs of anaplasmosis was between 42 and 60 days, which according to DE ANDRADE et al. (2004) is within the incubation period of *A. marginale* and within a possible immunological window for the parasite. Additionally, once the animal was infected, it remained infected until the end of the experiment. These findings corroborate those of LIMA et al. (2019), who observed the persistence of *A. marginale* infection in buffalo and cattle calves through an experimental study.

In addition, a significant decrease was observed in the mean globular the course of the study ($P < 0.01$), which was higher in the animals assessed between 1 and 20 days and lower in those assessed between 42 and 60 days. VG reflects the presence or absence of anemia; however, it should always be correlated with the animals' clinical signs, as dehydration, for example, can falsely elevate this test.

The experimental animals were calves aged between 1 and 60 days, so the response of adult animals cannot be compared. However, the older the animals, the higher was the percentage of infection, with a peak at 47 days. Furthermore, if we confirmed that there is a rickettsial linearity in the progression line shown in figure 2, with a tendency to plateau at the end of the study, this shows that the area is one of enzootic stability.

The peak in parasitemia at 47 days may be due to the incubation period of *A. marginale* of ≥ 2–4 weeks, and DE ANDRADE et al. (2004) reported that this depends on the sensitivity of the host and the level of rickets, as well as the persistence of colostral antibodies as a factor of resistance to *A. marginale*infection. MADRUGA et al. (1985) reported the data on average parasitemia during the experimental period, showing the importance of humoral immunity in resistance to anaplasmosis in young animals aged 30–90 days. ALFONSO et al. (1996) also demonstrated this immune response occurring after the second day of experimental infection by *A. marginale*, with the onset of leukocytosis which disappeared approximately 30–35 days after infection, characterized by fever and weight loss. Compared to the animals in the present study, they fall into the age group susceptible to rickets elevation, thereby having a possible immunological window for the action of the parasite.

Diagnosis using PCR is more sensitive than blood smears, and this procedure is indicated for detecting animals with low parasitemia. However, in this study, no significant difference, but rather a gradual increase in diagnosis, was observed between the techniques (blood smear and nPCR). This was possibly because the parasitemia in most of the animals studied was in a linear progression of infection, which contributed to the detection of the agent.

Results obtained from previous studies using PCR report a prevalence of 98.6% (1627/1650) of *A. marginale* in the animals in Rondônia (BRITO et al., 2010). SOUZA et al. (2013), studying cattle from the Piauí dairy basin, found a prevalence of 76.2% (154/202) for *A. marginale* using molecular analysis. Both studies have similar data to that found in the present study (95.8%). JAIMES-DUEÑEZ et al. (2017), using PCR, found infection rates of 59.3% (275/464) for *A. marginale* in cattle in Colombia. Younger animals are more likely to recover from *A. marginale* infection because they have a more active hematopoietic system compared to adult animals, which can favor their recovery (DE ANDRADE et al., 2004). This may explain the fact that; although, the average parasitemia was high in the experimental animals, and most of the animals showed clinical signs at some point during the experiment, none of them died.

The results presented here are similar to a study in Botucatu, São Paulo, in which mixed-breed calves aged up to 1 year had *A. marginale* as the main clinical disease agent, causing economic losses (GONÇALVES et al., 2011). In the present study, when investigating hemoparasites in blood smears, only two animals showed mixed infection by *A. marginale* and *B. bigemina,* with only one *B. bigemina* merozoite being found on each slide.

BAHIA (2021) reported that out of 300 animals examined, the presence of *A. marginale* wasdetected in 28.86% of the blood smears and 55.66% by nPCR. The studies can be correlated due to the similarities and prevalence of clinical disease associated with *A. marginale* infection. In addition, the sick animals were medicated with oxytetracycline and imidocarb dipropionate; however, the authors draw attention to the fact that despite clinical recovery from the disease, the animals may remain carriers of the agent.

Notably; although, it was not the aim of this study to evaluate the treatment used on the property

studied, it could be seen that the sick animals were treated with the same drugs mentioned above, thereby yielding similar results in terms of the permanence of *A. marginale* in the recovered animals. These results were observed via blood smear and ratified by nPCR; therefore, it can be concluded that these animals are carriers of the agent.

CONCLUSION

Of the 24 calves examined, none died and 21 showed symptoms at some point during the study. Notably, no significant difference of findings was observed between blood smears and nPCR, probably due to the medium and high parasitemia of the animals studied.

In addition, the highest frequency of ricketsemia occurred from the age of 20 days and increased up to 60 days, with a peak on Day 47. Clinical signs and a decrease in globular volume were correlated with an increase in parasitemia. In this sense, these results can be used by veterinarians who treat cattle in epidemiological conditions similar to those of this study.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest in this article.

AUTHORS' CONTRIBUTION

NSSS, JDBN, and DHSL conceived and designed the experiments, and critically reviewed the manuscript. MVM, MJCC, MHSS, NANJ, and EMJ conducted the experiment. MDC, MVM, IMA, and BAB conducted the laboratory analyses. PCM conducted the statistical analysis of the experimental data. MVM, MJCC, MHSS, NANJ, and EMJ drafted the manuscript. All the authors approved the final version of the manuscript.

BIOETHICS AND BIOSAFETY COMMITTEE APPROVAL

This study was approved by the Ethics Committee for the Use of Animals at the Universidade Federal do Pará (UFPA) (CEUA/UFPA), under the protocol number 5264261020.

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