

Investigation of *Anaplasma marginale*, *Babesia bovis*, *Babesia bigemina* and *Trypanosoma vivax* in the brain and spleen of dairy cows of Rio Grande do Sul

Investigação de *Anaplasma marginale*, *Babesia bovis*, *Babesia bigemina* e *Trypanosoma vivax* em cérebro e baço de vacas leiteiras no Rio Grande do Sul

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Highlights

The agents of cattle tick fever are present in the region studied.

Anaplasma marginale was the prevalent agent.

Trypanosoma vivax was not found in the investigated samples.

Abstract

Cattle tick fever and bovine trypanosomosis are diseases that negatively impact cattle production in Brazil, causing considerable productive losses and animal deaths. In this study, the objective was to investigate the presence of the four hemoparasites that mostly affect dairy herds (*Anaplasma marginale*, *Babesia bovis*, *Babesia bigemina* and *Trypanosoma vivax*) using the real-time quantitative polymerase chain reaction (qPCR) technique in discarded dairy cows in the northwestern region of Rio Grande do Sul, in southern Brazil. For this purpose, brain and spleen tissue biopsies were collected at a local

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slaughterhouse from 64 dairy cattle from this region. DNA extraction from the samples was performed using the Brazol[®] reagent, their quantity and purity were obtained through spectrophotometer analysis with NanoDrop[™]. In the brain samples, DNA detection was performed for *T. vivax* and *B. bovis*, and in the spleen, the DNA of the four agents was verified. From a total of 64 brain samples analyzed, 14.06% were positive for *B. bovis*. For a total of 64 spleen samples, *B. bovis* was detected in 17.19 % of the samples, while 29.69 % tested positive for *B. bigemina* and 37.50 % for *A. marginale*. Regarding *T. vivax*, none of the analyzed samples resulted in a positive diagnosis in both tissues. Thus, the findings highlighted that *B. bovis*, *B. bigemina* and *A. marginale* are present in the dairy cattle herd of the northwestern regions of Rio Grande do Sul, with a higher percentage of *A. marginale*; on the other hand, *T. vivax* was not diagnosed in the present study.

Key words: Cattle. Hemoparasitosis. Slaughterhouse. qPCR.

Resumo

A Tristeza Parasitária Bovina (TPB) e a tripanossomose bovina são enfermidades que impactam a produção bovina de forma negativa no Brasil, causando perdas na produção e morte de animais. Neste estudo, o objetivo foi investigar os quatro hemoparasitos que mais acometem o rebanho bovino (*Anaplasma marginale*, *Babesia bovis*, *Babesia bigemina* e *Trypanosoma vivax*) pela técnica de qPCR, em vacas leiteiras de descarte na região noroeste do Rio Grande do Sul. Para isso, foram coletados, em abatedouro, fragmentos de cérebro e de baço de 64 bovinos leiteiros dessa região. A extração do DNA das amostras foi feita por meio do reagente Brazol[®], e a quantidade e a pureza das mesmas, foi identificada por meio de espectofotometria. Nas amostras de cérebro foi realizada a pesquisa de DNA para *T. vivax* e *B. bovis*, enquanto no baço, investigou-se DNA dos quatro agentes. Das 64 amostras de cérebro analisadas, 14,06 % foram positivas para *B. bovis*. Nas 64 amostras de baço, *B. bovis* foi detectada em 17,19 %, enquanto 29,69 % testaram positivas para *B. bigemina* e, 37,50 %, para *A. marginale*. Em relação ao *T. vivax*, nenhuma resultou positiva em ambos os tecidos. Assim, os achados evidenciaram que *B. bovis*, *B. bigemina* e *A. marginale* estão presentes no rebanho bovino leiteiro, tendo maior percentual de *A. marginale* na região investigada; por outro lado, *T. vivax* não foi diagnosticado no presente estudo.

Palavras-chave: Bovino. Hemoparasitoses. Abatedouro. qPCR.

Introduction

Among the most frequently identified and reported hemoparasites in Brazil are *Anaplasma marginale*, *Babesia bovis* and *Babesia bigemina*. These hemoparasites are the agents responsible for cattle tick fever, which is directly linked to losses in meat and milk production (P. M. Gonçalves, 2000; M. B. Almeida et al., 2006; V. M. M. Costa et al., 2011; R. C. Gonçalves et al., 2011). The most

relevant pathogenic aspect of these agents is marked anemia, leading to high mortality rates in non-immunized cattle herds (Kessler & Schenk, 1998). Another protozoan that has been diagnosed in the Brazilian cattle herd and associated with clinical signs and financial losses similar to those caused by cattle tick fever, is *Trypanosoma vivax* (A. S. Silva et al., 2009; Cuglovici et al., 2010; Cadioli et al., 2012; Ono et al., 2017; Pereira et al., 2018).

Cattle tick fever is an important disease that has long been known in Brazil (Fonseca & Braga, 1924) and negatively impacts animal production (Dierings & Wilmsen, 2021). Babesiosis in cattle is usually caused by a protozoan of the genus *Babesia*. In Brazil both species, *B. bovis* and *B. bigemina* are normally found, which has the cattle tick (*Rhipicephalus microplus*) as its main vector (Trindade et al., 2011). Erythrocytes infected with *B. bovis* adhere to capillary endothelial cells of the brain and kidneys, and often the clinical symptoms are related to injuries on these organs, while *B. bigemina* distributes itself through circulation, causing symptoms consistent with hemolytic anemia (Gallego-Lopez et al., 2019). The rickettsia *A. marginale*, is the causative agent of anaplasmosis, sharing the same biological vector as babesiosis. In addition, mechanical transmission by hematophagous dipteran and contaminated fomites is often observed (Trindade et al., 2011). *Anaplasma marginale* is an obligate intracellular parasite, found in the erythrocyte membrane, making hemolytic anemia its main symptom (Kocan et al., 2010).

Bovine trypanosomosis is a disease caused by a protozoan parasite of the genus *Trypanosoma*. *Trypanosoma vivax* is considered the most pathogenic and important for cattle species (Gardiner, 1989; Germano et al., 2018). In Brazil, mechanical transmission is often observed by the bite of hematophagous insects such as tabanids and stable flies, as well as by needles contaminated with infected blood (R. A. M. S. Silva et al., 2002; Batista et al., 2008; Dagnachew & Bezie, 2015; Ono et al., 2017).

Several techniques are used for the identification of these parasites, however,

many of those techniques are less sensitive at low degrees of parasitemia, such as the blood smear and parasite count using a microhematocrit (Wells, 1984; Gardiner & Mahmoud, 1992). For this reason, detection through molecular techniques, such as PCR (Polymerase Chain Reaction), has been widely used and spread, for the detection of agents even in chronic infections, characterized by low parasitemia (P. J. Almeida et al., 1997; R. A. M. S. Silva et al., 2002; Guevara et al., 2011; L. R. Santos et al., 2016; Pereira et al., 2018). In this context, the objective of this study was to investigate the presence of *A. marginale*, *B. bovis*, *B. bigemina* and *T. vivax* by real-time quantitative PCR (qPCR) technique, in fragments of the brain and spleen of dairy cows discarded in the northwest region of Rio Grande do Sul, in southern Brazil.

Materials and Methods

Tissue samples were obtained from 64 dairy cows discarded, predominantly Holstein, at a state-inspected slaughterhouse, coming from 34 different dairy farms located in 21 municipalities in the northwest of Rio Grande do Sul (Table 1). The collections were made randomly, between the months of February and March 2022, on the slaughter line, along with the corresponding veterinary inspection. For detection of the agents, 1 cm³ fragments of brain and spleen were collected from each animal, packed in aluminum foil, and immediately stored in a refrigerated box (approximately 4°C) for transport, in order to reduce as much as possible, the degradation of the genetic material. Afterwards, they were kept frozen at -20 °C for later analysis in the laboratory.

Table 1

Number of animals and properties within the municipalities of the northwestern region of Rio Grande do Sul, in southern Brazil, during February and March 2022

Municipalities	Nº of properties	Nº of animals
Água Santa	1	1
Cerro Largo	2	4
Colorado	3	4
Coqueiros do Sul	1	2
Coxilha	1	2
Getúlio Vargas	1	1
Gramado dos Loureiros	1	1
Ibiraiaras	1	3
Marau	2	2
Mato Castelhano	3	8
Maximiliano de Almeida	1	2
Paim Filho	2	3
Ronda Alta	1	4
Roque Gonzales	2	4
São João da Urtiga	1	4
São Luiz Gonzaga	1	4
São Pedro do Butiá	2	3
Sertão	2	3
Três Palmeiras	1	1
Vila Langaro	3	3
Vila Maria	2	5
TOTAL	34	64

To perform the molecular diagnosis by qPCR, DNA was extracted using the Brazol® reagent (LCG Biotecnologia, BR), using 100 mg of each tissue sample collected. Initially, each fragment was macerated with the aid of a pestle, to promote tissue cell breakdown, in an Eppendorf tube containing 333 µL of Brazol®. The next extraction steps were carried out following the manufacturer's instructions, using 250 µL of chloroform, 500 µL of absolute ethanol, and 500 µL of 70 % ethanol. The DNA obtained during the process was eluted in

30 µL of ultra-pure water. The quantity and purity of each sample were obtained through spectrophotometer analysis (260/280 nm ratio) with NanoDrop™ (Thermo Fisher Scientific, USA). Finally, DNA was diluted in ultra-pure water at a final concentration of 20 ng/µL to perform molecular diagnostics and frozen at -20 °C.

In the brain sample, DNA detection was performed by qPCR for *T. vivax* and *B. bovis*, while, for sample spleen, for *T. vivax*, *B. bovis*, *B. bigemina* and *A. marginale*. Specific

primers were used for each of the studied species (Table 2). Furthermore, the GAPDH gene was used as a control of the integrity of the genetic material, for all samples studied (Zhao et al., 2016). For each sample, reactions were performed in duplicate, using 2 µL of primer (1 µL forward; 1 µL reverse), 2 µL of DNA (final concentration of 40 ng), 5 µL of Go Taq qPCR Master Mix (Promega) and 1 µL of ultra-pure water, obtaining a final volume

of 10 µL for each reaction. As a positive control for *T. vivax*, DNA from experimentally infected cows from previous studies was used (Bastos et al., 2020a). For other agents, DNA from experimentally infected and splenectomized cows was used, provided by other researchers. As a negative control, ultra-pure water was used in the same final volume of the reaction.

Table 2
Sequence of primers used in qPCR to detect the listed hemoparasites

Agent	Primer	Product size (pb)	Reference
<i>Trypanosoma vivax</i>	TvivF - 5'-AATGGCTTCTCCATTGGGTTC-3' TvivR - 5'-ATGGAGCAGGCAAAGAGACC-3'	210	Madruga et al. (2006)
<i>Babesia bigemina</i>	BbigF - 5'-GCAAACGCTTTGCAAACACC-3' BbigR - 5'AGCTAGCAGCTCTTGATTCTACC-3'	160	Drawn (access GenBank GQ214234)
<i>Babesia bovis</i>	BbovF - 5'-TGTTCTGGAAGCGTTGATTC-3' BbovR - 5'-AGCGTGAAAATAACGCATTGC-3'	88	Buling et al. (2007)
<i>Anaplasma marginale</i>	AnapF - 5'-AAGGCGAGGAGCTGTTTAAG-3' AnapR - 5'-CTACTGCCTCACAAAGGACGA-3'	104	Bacanelli et al. (2014)
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH F - 5'-GATGGTGAAGTCCGAGTGAAC-3' GAPDH R - 5'-GTCATTGATGGCGACGATGT-3'	100	Zhao et al. (2016)

The qPCR analysis was performed using CFX Opus 96 (Bio-Rad) in two steps (denaturation and annealing/extension), individually for each primer. All reactions started with an activation cycle of the Promega Gotaq enzyme at 95 °C for 60 s, followed by 39 cycles at 95 °C for 15 s and at 60 °C for 60 s to *T. vivax*; 39 cycles at 61 °C for 30 s and at 72 °C for 15 s to *B. bigemina*; 39 cycles at 64 °C for 30 s and at 72 °C for 30

s to *B. bovis* and; 39 cycles at 54 °C for 15 s to *A. marginale*, for denaturation and annealing/extension, respectively. Finally, the melting curve was analyzed considering a variation of 0.5 °C of the positive sample, to determine the specificity of reactions. Based on the results obtained, the percentages of positive and negative samples were calculated for each agent individually, as well as for cases of co-infection.

Results and Discussion

Under these analysis conditions, the melting temperatures found were: 81 °C for *A. marginale*, 78,5 °C for *B. bovis*, 83 °C for *B. bigemina*, and 87 °C for *T. vivax*. Minimal DNA detection for *A. marginale* was performed as described by Bacanelli et al. (2014) and for *B. bovis*, as described by Buling et al. (2007). For *B. bigemina* and *T. vivax* a serial dilution was performed, in which the minimum detection was 0.075 ng and 0.00075 ng, respectively.

According to the results obtained by qPCR, of the 64 brain samples analyzed, 14.06 % (9/64) were positive for *B. bovis*, while none of the analyzed samples detected *T. vivax* (Table 3). In the spleen samples, *B. bovis* was detected in 17.19 % (11/64) of the samples, while 29.69 % (19/64) were positive for *B. bigemina* and 37.50 % (24/64) for *A.*

marginale. As in the brain, the spleen also did not yield positive samples for *T. vivax*. In addition, when the spleen samples were analyzed, the presence of more than one agent was observed in 15.62 % (11/64) of the samples, with *B. bovis* and *A. marginale* being detected in 10.94 % (7/64) of them. Concomitant infection of *B. bovis* and *B. bigemina*, as well as the co-infection by *B. bigemina* and *A. marginale* were identified in only 1.56 % (1/64) of the animals analyzed. A total of 3.12 % (2/64) of the analyzed samples showed the presence of all three agents of cattle tick fever (*B. bovis*, *B. bigemina*, and *A. marginale*) (Table 4). Considering the municipalities investigated, in 71.43 % (15/21) at least one of the agents was observed, and in two of these, Paim Filho and Roque Gonzales, *A. marginale*, *B. bovis* and *B. bigemina* were detected (Table 5).

Table 3

Absolute and relative frequencies of detection of *B. bovis* and *T. vivax* obtained by qPCR in brain samples from dairy cows discarded in the northwestern region of Rio Grande do Sul, in southern Brazil, during February and March 2022

Agent	Absolute Frequency	Relative Frequency
<i>B. bovis</i>	9/64	14.06 %
<i>T. vivax</i>	0/64	0.00 %

Table 4

Absolute and relative frequencies of detection of *A. marginale*, *B. bovis*, *B. bigemina* and *T. vivax* obtained by qPCR in spleen samples from dairy cows discarded in the northwestern region of Rio Grande do Sul, in southern Brazil, during February and March 2022

Agent	Absolute Frequency	Relative Frequency
<i>A. marginale</i>	24/64	37.50 %
<i>B. bigemina</i>	19/64	29.69 %
<i>B. bovis</i>	11/64	17.19 %
<i>B. bovis</i> and <i>A. marginale</i>	7/64	10.94 %
<i>B. bovis</i> and <i>B. bigemina</i>	1/64	1.56 %
<i>B. bigemina</i> and <i>A. marginale</i>	1/64	1.56 %
<i>B. bovis</i> , <i>B. bigemina</i> , and <i>A. marginale</i>	2/64	3.12 %
<i>T. vivax</i>	0/64	0.00 %

Table 5

Distribution, of *A. marginale*, *B. bovis*, *B. bigemina* and *T. vivax*, by municipality investigates, obtained by qPCR in spleen and brain samples from dairy cows discarded in the northwestern region of Rio Grande do Sul, in southern Brazil, during February and March 2022

Municipalities	<i>A. marginale</i>	<i>B. bovis</i>	<i>B. bigemina</i>	<i>T. vivax</i>
Água Santa	1	0	0	0
Cerro Largo	0	0	1	0
Colorado	1	0	2	0
Coqueiros do Sul	0	0	0	0
Coxilha	1	1	0	0
Getúlio Vargas	1	0	0	0
Gramado dos Loureiros	0	0	0	0
Ibiraiaras	0	0	0	0
Marau	0	0	0	0
Mato Castelhano	5	0	2	0
Maximiliano de Almeida	0	1	0	0
Paim Filho	3	2	1	0
Ronda Alta	2	0	1	0
Roque Gonzales	1	1	4	0
São João da Urtiga	3	3	0	0
São Luiz Gonzaga	3	3	0	0
São Pedro do Butiá	0	0	2	0
Sertão	2	0	0	0
Três Palmeiras	0	0	0	0
Vila Langaro	0	0	2	0
Vila Maria	1	0	4	0
TOTAL	24	11	19	0

The majority of the studies reported in Rio Grande do Sul, aiming the identification of the cattle tick fever agents (*A. marginale*, *B. bovis* and *B. bigemina*), are focused on the center-south region (Rodrigues et al., 2005; M. B. Almeida et al., 2006; Schild et al., 2008; T. M. Silva et al., 2018). However, a previous study that evaluated the economic impact of cattle tick fever in the northwest of Rio Grande do Sul, showed an approximate loss of 246.7 million dollars, over a 3-year period (Vieira & Costa, 2006). Furthermore, it can be observed that the disease is prevalent in both tropical and subtropical regions of the country (H. Q. Santos et al., 2001; M. B. Almeida et al., 2006; R. C. Gonçalves et al., 2011; Gris et al., 2016; T. M. Silva et al., 2018; T. F. Silva et al., 2021), causing losses that can reach 3.5 billion dollars annually (M. O. Costa et al., 2021; T. F. Silva et al., 2021).

In the southern regions of Brazil, there are few reported studies on the prevalence of bovine trypanosomosis, however, *T. vivax* has been identified in a blood smear from a suspected case in the municipality of São Pedro in Rio Grande do Sul, in the central region of the state, which was further confirmed by PCR (A. S. Silva et al., 2009), there are no reports of other studies with this parasite in the state. Our data did not reveal any detection of *T. vivax* in the studied region, which suggests a low prevalence of this protozoan parasite in the cattle in this region. Furthermore, in the brain, the protozoan may not have been detected, as it may not have crossed the blood-brain barrier and invaded the cells of the brain parenchyma (D'Archivio et al., 2013). However, in other Brazilian regions, bovine trypanosomosis has become a serious problem for cattle production, since

outbreaks are becoming more and more frequent. In the state of Goiás, a 39.62 % drop in average milk production was observed in 24 properties evaluated with outbreaks of bovine trypanosomosis caused by *T. vivax* (Bastos et al., 2020b). In Minas Gerais, Carvalho et al. (2008), reported a 27 % reduction in milk production and a 45 % decrease in pregnancy rates during an outbreak of this disease. It was also observed a sharp reduction in daily milk production in cows infected with *T. vivax* in São José, Paraíba, from 15 liters per day to an average of 2 liters per day (Batista et al., 2008).

Considering the data found in this study, it can be observed a higher occurrence of *A. marginale* (37.5 %) among the culled animals diagnosed in the northwest of Rio Grande do Sul. This data is in agreement with the findings of Vieira and Costa (2006), who analyzed the economic impact of cattle tick fever in the same region and reported a higher rate of anaplasmosis recurrence in lactating animals, among all the clinical cases that were investigated. A retrospective study in the southern region of Rio Grande do Sul, based on 221 outbreaks of cattle tick fever, showed 29.41% of positive cases for *A. marginale* (M. B. Almeida et al., 2006), once again highlighting a high percentage of this agent in cattle herds.

When evaluating factors related to the occurrence of *A. marginale* and other hemoparasites in 401 cattle in India, Das et al. (2022) observed 63 animals positive for *A. marginale* by PCR. The highest prevalence of the agent was in animals aged 3 to 5 years, and the lowest prevalence was observed in animals under 1 year of age. *A. marginale* was observed more frequently in females than in

males and in Holstein Friesian animals than in other breeds. Cases of babesiosis and trypanosomosis were not detected. These data are consistent with those observed in the present study, as all animals tested were discarded females, predominantly of the Holstein breed. This suggests that factors such as age, sex, and breed may be related to a higher occurrence of *A. marginale*. Furthermore, mechanical transmission by hematophagous dipterans of the genus *Tabanus* and *Stomoxys* is the main mode of *A. marginale* spread in South America (Kocan et al., 2003), and stable fly outbreaks with recurrence are reported in Rio Grande do Sul (Barros et al., 2023). Considering these factors and the 37.50 % percentage of *A. marginale* found in this study, it can be suggested that this agent occurs frequently, indicating its current presence in the studied region.

Babesia bigemina, was the second most detected agent, totaling 29.69 % of cases, showing a higher percentage when compared to the study by M. B. Almeida et al. (2006) who observed the disease in 4.90% of outbreaks. However, in this same study, 14.93 % of the cases analyzed were also positive for babesiosis, but the species was undefined, which may be suggestive of a higher percentage of *B. bigemina*. One possible reason for the higher number of *B. bigemina* cases when compared to *B. bovis* cases is the transmission, since *B. bigemina* can be transmitted by nymphs and adults of ticks, whereas *B. bovis* is inoculated into cattle only by tick larvae (Callow & McGavin, 1963; Mahoney et al., 1973).

Red Blood Cells (RBCs) parasitized with *B. bovis*, adhere to capillary endothelial brain cells causing nervous symptoms,

besides generalized clinical signs (Gallego-Lopez et al., 2019). *Babesia bovis* was investigated in fragments of brain and splenic tissue, being found in 14.06 % and 17.19 %, respectively. All samples positive for the brain were also positive in the spleen, however, 2 cases with positive diagnosis in the splenic tissue resulted in a negative diagnosis in the brain. This is probably due to parasitized erythrocytes being destroyed by macrophages, as the parasite is intracellular, and fragments of it may remain in the spleen when destroyed (Brown et al., 2006). M. B. Almeida et al. (2006) observed *B. bovis* in 41.1 % of the outbreaks in a retrospective study in the south of Rio Grande do Sul, this percentage can be even higher, since in some cases, the genus *Babesia* was not identified.

In another study conducted in the central region of Rio Grande do Sul, which used histochemical methods to diagnose *B. bovis* in 12 animals suspected of cerebral babesiosis, the agent was found in 7, mainly observed in the capillaries of the gray matter of the encephalon. However, the parasite was also found in the capillaries of the white matter and in some larger vessels (T. M. Silva et al., 2018). Therefore, it is possible to suggest that the tissue sample used for diagnosis in the present study might not have contained the parasite, resulting in a lower number of positive samples. Furthermore, since these were discarded animals in slaughterhouses, their clinical history is unknown, and they could have presented a chronic infection of the disease. At this stage of the disease, there are fewer infected erythrocytes and consequently fewer circulating parasites (Brown et al., 2006), or alternatively, the animals could have tested negative for the agent.

The milk production chain in Rio Grande do Sul is significantly influenced by regional characteristics. The northwest region of the state, contributing to 66.5% of the state's milk production, houses the largest dairy herd (Empresa de Assistência Técnica e Extensão Rural [EMATER], 2021). In contrast, extensive beef cattle farming is predominant in the southern region, while the northern region emphasizes dairy cattle production within a semi-extensive production system (G. de S. Silva et al., 2014). Hence, the higher incidence of *A. marginale* in the northern region of the state can likely be attributed to the prevalent production system. In this system, animals spend more time stabled and have increased contact with stable flies. Conversely, the southern region, characterized by an extensive production system, exposes animals to greater contact with cattle ticks, the biological vector for *B. bovis* and *B. bigemina* (Trindade et al., 2011).

Also, regarding the agents of cattle tick fever, cases of co-infection were found, where 10.94 % of the cases, the presence of both *B. bovis* and *A. marginale* was observed, while in 1.56 % both *B. bovis* and *B. bigemina* or *B. bigemina* and *A. marginale* was detected, and in 3.12 % of the samples the three agents were present (*B. bovis*, *B. bigemina* and *A. marginale*). Previous reports have described more than one agent of cattle tick fever, with a rate of 9.50 % for *A. marginale* and *Babesia* sp. (M. B. Almeida et al., 2006). In addition, the combined presence of all four agents (*T. vivax*, *A. marginale*, *B. bovis* and *B. bigemina*) has been reported by Côte d'Ivoire (Yeó et al., 2017). The observed co-infection results in this study underscore the significance of implementing specific therapeutic protocols

for both diseases treatment and vector control, particularly given the presence of all cattle tick fever agents in the region. Chemotherapy often involves a combination of compounds, considering that these agents have distinct pathogenic mechanisms (P. M. Gonçalves, 2000; Kocan et al., 2010).

Conclusion

Our findings suggest that *B. bovis*, *B. bigemina* and *A. marginale* can be present in dairy cattle herds in the northwest region of Rio Grande do Sul, where the most diagnosed agent was *A. marginale*. The protozoan *T. vivax* was not identified in this study; however, further research should be conducted on this agent in the southern region of Brazil.

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Conflict of interest

The authors declare they have no conflict of interest.

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