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Original Article

Characterization of cattle tick fever in calves from the northwestern region of Minas Gerais, Brazil

Caracterização do complexo da Tristeza Parasitária Bovina em bezerros da região noroeste de Minas Gerais, Brasil

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Abstract

The present study aimed to characterize the importance of the *Anaplasma marginale, Babesia bovis* and *Babesia bigemina* in the genesis of cattle tick fever (CTF) among dairy calves in the northwest of Minas Gerais, Brazil. Blood samples from 300 calves were collected, followed by DNA extraction and nested PCR using oligonucleotide primers to amplify fragments of the semi-nested for the *msp5* gene (*A. marginale*), *sbp-4* (*B. bovis*) and *rap-1a* (*B. bigemina*) Among the examined calves, the prevalence of *A. marginale* was 55.6% (n=167/300), *B. bovis* was 4.0% (n=12/300) and *B. bigemina* was 15.3% (n=46/300), by PCR techniques. Parasitic forms of *A. marginale* and *B. bigemina* were found in 36,3% and 2,6% of the blood smears while *B. bovis* was not detected. There was a statistical difference between the positivity of infected animals in the age groups 1 (10-70 days) and (>70-300 days) for *A. marginale* and *B. bigemina.* A total of 15 calves with the classic symptoms of disease were examined, and the samples obtained were confirmed as a simple infection by *A. marginale* through semi-nested PCR. These results confirm bovine anaplasmosis as the primary cause of CTF among the calves of dairy cattle within the studied area.

Keywords: Calves, tick borne diseases, hemoparasites, Brazil.

Resumo

O presente estudo teve como objetivo caracterizar a importância de *Anaplasma marginale*, *Babesia bigemina* e *Babesia bovis* na gênese da tristeza parasitária bovina em bezerros leiteiros do noroeste de Minas Gerais. Foram coletadas 300 amostras sanguíneas de bezerros, seguidas por extração de DNA e Nested- PCR utilizando oligonucleotídeos iniciadores que amplificam fragmentos dos genes *sbp-4* (*B. bovis*) e *rap-1a* (*B. bigemina*) e a Semi-Nested para o gene *msp5* (*A. marginale*). A prevalência de *A. marginale* foi 55,66% (167/300), *B. bigemina*, 15,33% (46/300) e *B. bovis* 4,0% (12/300) dos bezerros examinados. Formas parasitárias de *A. marginale* and *B. bigemina* foram encontradas em 36,33% e 2,66% dos esfregaços sanguíneos, enquanto *B. bovis* não foi detectado. Houve diferença estatística entre as prevalências de animais infectados nas faixas etárias 1 (10-70 dias) e 2 (>70-300 dias). Um total de 15 animais com sintomas clássicos da doença foram examinados, e as amostras foram confirmadas como uma infecção simples por *A. marginale* através da Nested-PCR. Esses resultados confirmam a anaplasmose bovina como a principal agente da tristeza parasitária bovina nos bezerros do rebanho estudado.

Palavras-chaves: Bezerros, doenças transmitidas por carrapatos, hemoparasitos, Brasil.

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Introduction

The state of Minas Gerais stands out in the national context as the largest milk producer in Brazil. According to the Brazilian Institute of Statistics and Geography, the state had an estimated population of 3.4 million milked cows in 2017, with approximately 8.9 billion liters of milk being produced in Minas Gerais State (IBGE, 2017). However, tick-borne diseases are limiting factors for calf rearing among rural properties in the region (Ribeiro et al., 1984).

Cattle tick fever (CTF) is caused by a complex of frequent diseases among cattle in Brazil (Gonçalves, 2000). The agents causing this clinical manifestation include the protozoa *Babesia bovis* and *Babesia bigemina*, causing babesiosis, and rickettsia *Anaplasma marginale*, which causes anaplasmosis (Guglielmone, 1995). The agents responsible for bovine babesiosis in Brazil, are transmitted by the *Rhipicephalus microplus* tick, while the rickettsia that causes anaplasmosis can be biologically transmitted by *R. microplus*, mechanically transmitted by hematophagous arthropods such as flies and mosquitoes, fomites, and transmitted via the transplacental route (Kessler, 2001; Kocan et al., 2004; Silva et al., 2015).

When affected by anaplasmosis or babesiosis, animals exhibit clinical signs such as anorexia, shivering, tachycardia, tachypnea, reduced ruminal movements, prostration, reduced lactation, anemia, and jaundice. In the case of babesiosis may occurs hemoglobinuria and in the *B. bovis* infection, the animal may also show neurological signs, such as incoordination, staggering gait, pedal movements, and aggressiveness (Almeida et al., 2006; Costa et al., 2011; Oliveira et al., 2018; Silva et al., 2018).

In Brazil, the largest of the territory is considered of enzootic stability (Souza et al., 2000; Juliano et al., 2007; Brito et al., 2010, 2013). However, areas such as the dry region of Sergipe, dry region of Paraiba, in the central south of Paraná and extreme south of Rio Grande do Sul, are regions of enzootic instability. In this condition, younger animals possess low immunity against *A. marginale* and *Babesia* spp. Therefore, when they become infected at a later age, they may develop acute clinical symptomatology, which is associated with high mortality rates (Oliveira et al., 1992; Artiles et al., 1995; Marana et al., 2009; Costa et al., 2011).

The blood smear is the oldest method and is routinely used for diagnosing hemoparasites. In the acute phase of the disease, when parasitemia is high, parasites are easily detected in bovine erythrocytes (Böse et al., 1995). In carrier animals this method is less sensitive since the parasitemia is low. In this case, molecular tools using Polymerase chain reaction (PCR) increase the sensitivity (Al-Hosary, 2017).

PCR has been described as a useful tool in epidemiological studies presenting high specificity and sensitivity in the diagnosis of the etiological agents of CTF (Brito et al., 2010; Mosqueda et al., 2012; Giglioti et al., 2016; Romero-Salas et al., 2016). As such, the objective of the present study was to determine the prevalence and importance of *B. bigemina*, *B. bovis*, and *A. marginale* using the PCR and blood smear technique in calves from the northwestern region of Minas Gerais.

Material and Methods

Compliance with ethical standards

The present study was approved by the Council of Ethics in the Use of Animals (CEUA) of the Federal University of the Jequitinhonha and Mucuri Valleys (UFVJM) under protocol No. 060/2016. The research is in accordance with the precepts and norms of the National Council of Control of Animal Experimentation (CONCEA) in Brazil.

Sample determination

Animals from four dairy farms located in the northwestern region of Minas Gerais state, Brazil, were randomly selected. The properties were previously selected based on reports of clinical CTF cases from the producers within the last five years. Three farms located in the municipality of Unaí (FARM A, B and C) and one rural property located in Cabeceira Grande (FARM D) were selected. The description of the location of each farm (coordinates), farm size, total number of animals per herd, number of animals evaluated per farm, age groups, and breed of herd are described in Table 1.

Table 1. Description of calves of the dairy farms evaluated from the Northwest of Minas Gerais, Brazil.

Municipality	Farm (localization)	Farm Size	Total animal /farm	Animals examined	Group 1 (10-70 days)	Group 2 (>70-300 days)	Dairy breeds
Unaí	Farm A 16°06'46.6"S 47°05'49.8"W	240 hectares	800	100	30	70	Dutch; Gir; Jersey; Crosses
	Farm B 16°19'43.6"S 46°50'51.5"W	200 hectares	400	33	13	20	Dutch; Gir; Crosses
	Farm C 16°24'45.2"S 47°05'37.5"W	300 hectares	350	33	22	11	Dutch; Gir; Crosses
Cabeceira Grande	Farm D 16°06'46.6"S 47°05'49.8"W	450 hectares	1000	134	42	92	Dutch; Jersey; Kiw Cross

The calculation of the minimum sample size was determined by the following formula: $N = p.(100-p)Z^2/(d.p/100)^2$, where N = number of samples; p = expected prevalence; Z = trust rating; d = margin of error. The expected prevalence of 50% for CTF agents was estimated by a previous pilot. The confidence interval was 95% and the margin of error was 5%. Thus, the required sample size was at least 196 animals.

Animals

The analyzed herds comprised of several dairy breeds (Dutch, Gir, Jersey, Kiwi Cross and their crosses). Visits to the properties were conducted between January and September 2017. The evaluated animals were randomly selected according to the availability of the property. Two groups of calves were evaluated in the present study. Group 1 with animals aged 10 to 70 days and group 2 with animals older than 70 to 300 days.

Following the management adopted in each one of the properties, in Group 1 (10-70 days) all animals received colostrum in the first three days following birth, and subsequent suckling was performed through a baby bottle and individual buckets. In addition to milk, all calves received Tifton grass (*Cynodon* spp.) hay and concentrated rations for calves. Weaning of the animals was performed by body weight (i.e. when the animals reached a body weight between 70 kg and 100 kg), varying with the breeds of the animals. The Group 2 (> 70 – 300 days), consisted of weaned animals kept in *Brachiaria brizantha* grass, receiving corn silage and concentrated feed for heifers in troughs. These animals presented weights ranging from 100 kg to 220 kg according to the racial pattern. During the collection of biological samples, the animals were inspected for the presence of ectoparasites. Herds were vaccinated against brucellosis, foot-and-mouth disease (FMD), clostridiosis, rabies and Bovine Viral Diarrhea (BVD).

Sample collections and blood smear

Blood samples were collected from 300 calves of both sexes of group 1 (n = 107) and group 2 (n = 193). Sample collection were collected into tubes with anticoagulant ethylenediamine tetraacetic acid (EDTA) for subsequent extraction of DNA to perform the PCR. Blood smears were prepared, fixed with methanol, stained with Giemsa and microscopically observed for the detection of *A. marginale* and *Babesia* spp. in blood.

DNA Extraction

DNA extraction from whole blood was performed using the Wizard Genomic DNA Purification Kit (Promega, USA) according to the manufacturer's recommendations. The extracted DNA was quantified and stored in 100 µL aliquots in a freezer at -20 °C for subsequent PCR.

Molecular detection of *A. marginale*, *B. bovis*, and *B. bigemina*

Molecular diagnosis for *A. marginale* was performed using the semi-nested PCR technique for the *msp5* gene standardized by (Torioni De Echaide et al., 1998). According to the method of (Terkawi et al., 2011), nested PCR was adopted to amplify the fragments of *sbp-4* and *rap-1a* genes of *B. bovis* and *B. bigemina*, respectively. The primers used for the CTF agents as well as the sizes of amplified fragments are presented in Table 2.

Table 2. Primer sets specific for *A. marginale msp5, B. bovis sbp-4* and *B. bigemina rap-1a* genes using for molecular assays.

Genes	Etiological agent	Assays	Oligonucleotide primers	Product sizes
msp5	*A. marginale*	PCR	5- GCATAGCCTCCGCGTCTTTC-3	457 bp
			5 –TCCTCGCCTTGGCCCTCAGA-3	
		snPCR	5 -TACACGTGCCCTACCGAGTTA-3	344 bp
			5 –TCCTCGCCTTGGCCCTCAGA-3	
sbp-4	*B. bovis*	PCR	5-AGTTGTTGGAGGAGGCTAAT-3	907 bp
			5 -TCCTTCTCGGCGTCCTTTTC-3	
		nPCR	5 -GAAATCCCTGTTCCAGAG-3	503 bp
			5 -TCGTTGATAACACTGCAA-3	
*rap-1*a	*B. bigemina*	PCR	5-GAGTCTGCCAAATCCTTAC-3	879 bp
			5-TCCTCTACAGCTGCTTCG-3	
		nPCR	5-AGCTTGCTTTCACAACTCGCC-3	412 bp
			5-TTGGTGCTTTGACCGACGACAT-3	

PCR: Polimerase Chain Reaction; snPCR: semi-nested Polimerase Chain Reaction; nPCR: nested Polimerase Chain Reaction; bp: base pairs.

PCR products were analyzed by 1.5% agarose gel electrophoresis. For each reaction, a positive control and two negative controls (ultrapure water, HyPure™ Molecular Biology) were included. Positive controls were obtained from the blood of naturally infected cattle from the municipality of Seropédica, Rio de Janeiro, Brazil.

Statistical analysis

The frequencies of *A. marginale*, *B. bovis*, and *B. bigemina* positive animals between the group 1 (10–70 days) and group 2 (> 70–300 days) were compared by Fisher's exact or Chi-square test, with a confidence level of 95%. Statistical operations were performed with the aid of the statistical package R Foundation for Statistical Computing, version 3.6.2 (2019). The frequencies of *A. marginale*, *B. bigemina* and *B. bovis* positive animals between the farms were done by variance analyze (ANOVA) with 95% confidence level.

Results

Among examined animals, the prevalence of *A. marginale* was 55.66%, *B. bigemina* was 15.33%, and *B. bovis* was 4.0% by PCR. Parasitic forms of *A. marginale* and *B. bigemina* were found in 36,33% and 2,66% of the blood smears while *B. bovis* was not detected (Table 3).

Table 3. Prevalence of agents *Anaplasma marginale*, *Babesia bovis*, and *Babesia bigemina* in 300 calves from the Northwest of Minas Gerais, Brazil.

Municipality	*A . marginale*		*B. bigemina*		*B. bovis*	
	snPCR	Blood smear	nPCR	Blood smear	nPCR	Blood smear
Cabeceira Grande	42.53%	28.86%	12.68%	2.98%	4.0%	0%
Unaí	66.26%	43.97%	17.46%	2.40%	6.62%	0%
Total	55.66%	36.33%	15.33%	2.66%	4.0%	0%

Sn PCR - Semi nested PCR; nPCR - nested PCR.

The frequencies of CTF agent infections on each farm are shown in table 4. No statistical differences were found between the properties for *A. marginale* (p-*value*: 0.0521) *B. bigemina* (p-*value*: 0.6388) and *B. bovis* (p-*value*: 0.7760).

Table 4. Prevalence of agents *Anaplasma marginale*, *Babesia bigemina* and *Babesia bovis* in calves of dairy farm in the Northwest of Minas Gerais, Brazil.

Municipality	Farm identification	*A. marginale*	*B. bigemina*	*B. bovis*
Unaí	A	54.00% (54/100)	1.2% (12/100)	6.0% (06/100)
	B	90.90% (30/33)	15.15% (05/33)	6.06% (02/33)
	C	78.78% (26/33)	36.36% (12/33)	9.09% (03/33)
Cabeceira Grande	D	42.53% (57/ 134)	12.68% (17/134)	0.74% (01/134)
p- value		0.0521	0.6388	0.7740

Infection rates in the group 1 of calves were 41.12%, 26.16%, and 3.73% for *A. marginale*, *B. bigemina*, and *B. bovis*, respectively, while in the group 2, the prevalence was 63.73%, 9.32%, and 4.14% for the agents, correspondingly. A statistical difference was observed for *A. marginale* (p-*value*:0.0002575) and *B. bigemina* (p-*value*: 0.0002065) infection, while for *B. bovis* there was no difference (p-*value*: 1).

A total of 15 animals with clinical suspicion of CTF were examined at the time of site visits, and infection was confirmed only by *A. marginale*. Five animals were from Unaí (Farm A), while the teen animals were farm D from Cabeceira Grande. During the

inspection of the calves of each property observed ticks and blood flies (Horn fly, Stable fly), and according to farmers in periods of higher rainfall tabanids are observed.

The sick animals belonged to group 2 of the properties, with an age range of 70 to 300 days. Calves with anaplasmosis presented mainly fever, weight loss, pale mucosae and jundaice.

The producers reported that animals affected by the disease were commonly treated with several drugs, such as oxytetracycline at a concentration of 20 mg / kg and imidocarb dipropionate at 2.1 mg / kg predominating. Ivermectin application, at 1 mg / kg and bathing with the contact product COLOSSO PULVERIZAÇÃO® (cypermethrin 15.0 g, chlorpyrifos 25.0 g, and citronellal 1.0 g, Ouro Fino, Brazil), and a pour-on application of the systemic product FLUAZURON® (fluazuron 3.00 g and abamectin 0.50 g, Ouro Fino, Brazil).

Discussion

The frequency of positive for agents of CTF animals in PCR was higher than in blood smear. No parasitic forms of *B. bovis* were found in the blood smear. Fahrimal et al. (1992) reports that it is difficult to detect *B. bovis* infected carrier animals in the blood smear due to the low concentration of parasites present in the peripheral blood. According to Böse et al. (1995) the PCR technique is 100 times more sensitive than the blood smear technique, being indicated for the detection of animals with low parasitemia.

Higher infection rates of *A. marginale* than *Babesia* spp. were observed in the present study, although they have the same *R. microplus* biological vector, the intensive use of acaricides may be reducing the transmission rate of protozoa in the region.

As an important epidemiological factor for the establishment of higher prevalence of *A. marginale* is the persistence of reservoir infection. Consequently, Mechanical vectors (hematophagous flies and mosquitoes), fomites (surgical material and vaccination) may contribute to increased regional spread of *A. marginale* in herd. (Kessler, 2001; Reinbold et al., 2010; Silva et al., 2015).

Our results differ from previous studies that obtained prevalence values of 98.6 and 95.1% for *A. marginale* and *B. bovis*, respectively, in the state of Rondônia, in the Brazilian Amazon (Brito et al., 2010, 2013). In this study, the authors observed prevalence values of 76.2%, 52.5%, and 33.2% for *A. marginale*, *B. bigemina*, and *B. bovis*, respectively, in the Piauí dairy basin—an area of enzootic stability for rickettsia and enzootic instability for *Babesia* spp. Notably, (Jaimes-Dueñez et al., 2017) observed infection rates of 59.3%, 31.5%, and 13.8% for *A. marginale*, *B. bigemina*, and *B. bovis*, respectively, in cattle in Colombia, results which are similar to those described in the present study.

It was observed that calves from the group 2 had a higher prevalence of *A. marginale* infection, thus corroborating with authors who associate the increase in age with an increase in CTF prevalence (Ibrahim et al., 2013; Silva et al., 2015; Abdela et al., 2018). Low frequency of *B. bigemina* infection was observed in the same group. The use intensive of acaricides can be interfered on lower protozoan transmission rates.

The lower prevalence of *Anaplasma* infection in younger animals can be attributed to restricted grazing which reduces the probability of contact with vectors (hematophagous flies and ticks). In addition, the lower prevalence detected in young animals can be attributed to passive immunity transmitted during colostrum ingestion (Abdela et al., 2018). In addition, the tick life stage influences the transmission dynamics of *Babesia* spp. Since *B. bovis* and *B. bigemina* are transmitted by *R. microplus* larvae and nymphs, respectively (Bock et al., 2004), the degree of infestation of these stages in animals may have fluctuated over the months on the farms of northwestern Minas Gerais, thus affecting infection rates in the groups of calves.

Corroborating these results, Melo et al. (2001) found that over 90% of calves possessed antibodies against *A. marginale* within the first month of life, with these values dropping to a minimum of 13.6% from three to five months. However, due to the first infection, over 96% from six months of age were positive again due to acquired immunity.

The present results corroborate with other studies where the prevalence of clinical disease was primarily associated with simple infection by *A. marginale* (Gonçalves et al., 2011; Costa et al., 2011; Amorim et al., 2014). Botucatu, a city in São Paulo state, was part of a retrospective study of CTF cases from 1987 to 2007 in crossbred calves up to one year old, and *A. marginale* was the main agent of the disease, which caused regional economic losses (Gonçalves et al., 2011). In the northeast of Brazil, Costa et al. (2011) described the occurrence of 24 outbreaks in the interior of Paraiba, with 75% of cases being associated with simple infection by *A. marginale*, while (Amorim et al., 2014) detected *A. marginale* as the main agent found in CTF within the southern region of Bahia. The higher frequency of clinical anaplasmosis cases may also be associated with the broad genetic diversity of *A. marginale*, and studies report different pathogenic strains involved in disease outbreaks among cattle herds in the Americas (Almazán et al., 2008; Ruybal et al., 2009; Machado et al., 2015; Silva et al., 2015).

In the dairy farms studied, the reports are on clinical cases of anaplasmosis in animals of group 2 with age of 70 to 300 days. According to Zabel et al. (2018), anaplasmosis is most commonly observed in cattle over one year of age. In addition, cows in the final third of gestation and/or lactation may exhibit a immunosuppression and signs of acute anaplasmosis (Aktas & Özübek, 2017). However, the super acute anaplasmosis characterized by a high mortality rate within a few hours of the development of clinical signs is more frequently observed in dairy breeds (Abba et al., 2016).

Low rates of infection by *B. bigemina* and *B. bovis* were observed in calves from farms of the northwestern of Minas Gerais. Babesiosis outbreaks with high mortality rates have been described related to the transfer of animals from areas of instability to zones of enzootic stability where climatic conditions, farm management and intensive use of acaricides interfered in the development of the vector tick (Schild et al., 2008; Hue et al., 2013; Oliveira et al., 2018) In these case the vaccination has been an important method for preventing babesiosis outbreaks (Gohil et al., 2013).

The properties analyzed in the present study used drugs based on oxytetracycline and imidocarb dipropionate, and both drugs are effective in the treatment of bovine anaplasmosis. Following clinical recovery from the disease, animals remain carriers of the agent; thus, different protocols made with both drugs were not effective for the chemical sterilization of *Anaplasma* (Coetzee et al., 2005, 2006; Alberton et al., 2015). The recommended treatment protocols used were based only on imidocarb dipropionate for the treatment of CTF in the Northwest region of Minas Gerais state, and are inappropriate due to inefficiency in the treatment of bovine anaplasmosis. In this context, it is recommended to conduct an adequate diagnosis and adapt the treatments for tetracycline or oxytetracycline-based medicinal products. In addition, according to Doyle et al. (2007), imidocarb dipropionate has adverse effects such as salivation, tearing, tachypnea, and pain at the site of injection, and is more commonly used in association with *Babesia* spp. infection with *A. marginale*.

Conclusion

The agents of CTF infect calves in the Northwest region of Minas Gerais, Brazil. As such, treatment protocols adopted in this region should be recommended based on an adequate diagnosis, prioritizing the insertion of broad-spectrum antibiotics that are effective against bacterial infections due to the occurrence of *A. marginale*.

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