Serological occurrence for tick-borne agents in beef cattle in the Brazilian Pantanal

Ocorrência sorológica para agentes transmitidos por carrapatos em bovinos de corte no Pantanal Brasileiro

Inalda Angélica de Souza Ramos[1]; Victória Valente Califre de Mello[1]; Natalia Serra Mendes[1];
Diego Carlos de Souza Zanatto[1]; João Bosco Vilela Campos[2]; João Vitor Almeida Alves[2];
Gabriel Carvalho de Macedo[2]; Heitor Miraglia Herrera[2]; Marcelo Bahia Labruna[3];
Gener Tadeu Pereira[4]; Rosangela Zacarias Machado[1]; Marcos Rogério André[1]*

[1] Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, Jaboticabal, SP, Brasil

[2] Departamento de Parasitologia Veterinária, Universidade Católica Dom Bosco, Campo Grande, MS, Brasil

[3] Departamento de Medicina Veterinária Preventiva e Saúde Animal, Universidade de São Paulo, São Paulo, SP, Brasil

[4] Departamento de Ciências Exatas, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, Jaboticabal, SP, Brasil

Abstract

This study investigated the seropositivity for five different tick-borne agents, namely *Anaplasma marginale*, *Babesia bovis*, *Babesia bigemina*, *Coxiella burnetii*, *Anaplasma phagocytophilum,* and *Trypanosoma vivax* in beef cattle in the Brazilian Pantanal. The serum samples collected from animals (200 cows; 200 calves) were used in indirect enzyme-linked immunosorbent assays (iELISA) to detect IgG antibodies against *A. marginale*, *B. bovis*, *B. bigemina,* and *T. vivax,* and Indirect Fluorescent Antibody Test (IFAT) for detecting IgG antibodies against *C. burnetii* and *A. phagocytophilum*. No correlation was observed between seropositivity for *C. burnetii* and *A. phagocytophilum* with other agents whereas moderate correlation was observed for *A. marginale* x *B. bigemina* x *B. bovis.* Cows were more seropositive for *T. vivax* whereas calves were more seropositive for *B. bovis* and *B. bigemina*. The highest number of seropositive animals by a single agent was observed for *T. vivax* (15.2%). Co-seropositivity for *T. vivax* + *A. marginale* was higher in cows (25.5%) and for *T. vivax* + *B. bovis* + *B. bigemina* + *A. marginale* was higher in calves (57.5%). The high seropositivity correlation for *A. marginale* x *B. bovis* x *B. bigemina* is probably due to the presence of the tick biological vector, *Rhipicephalus microplus,* in the studied farms. Common transmission pathways, mediated by hematophagous dipterans and fomites, may explain the high co-seropositivity of cows for *A. marginale* and *T. vivax*. Low seropositivity to *C. burnetii* is probably due to the type of breeding system employed (extensive). Seropositivity for *A. phagocytophilum* in only one animal suggests the occurrence of a cross-serological reaction with another agent of the genus *Anaplasma*.

Keywords: Trypanosomiasis, Anaplasmosis, Babesiosis, Q-Fever.

Resumo

Este estudo teve como objetivo determinar a co-soropositividade para agentes transmitidos por carrapatos, como *Anaplasma marginale*, *Babesia bovis*, *Babesia bigemina*, *Coxiella burnetii*, *Anaplasma phagocytophilum,* e *Trypanosoma vivax* em bovinos de corte do Pantanal Brasileiro. Amostras de soro foram colhidas de 400 animais (200 vacas; 200 bezerros) e submetidas a Ensaios Imunoenzimáticos Indiretos (iELISA) para detecção de anticorpos IgG anti- *A. marginale*, anti- *B. bovis*, anti- *B. bigemina* e anti- *T. vivax,* e à Reação de Imunofluorescência Indireta (RIFI) para detecção de anticorpos IgG anti - *C. burnetii* e anti- *A. phagocytophilum.* Ausência de correlação foi vista entre os animais soropositivos para *C. burnetii* e *A. phagocytophilum* com os outros agentes e correlação moderada ocorreu entre *A. marginale* x *B. bigemina* x *B. bovis*. Vacas foram mais soropositivas que bezerros para *T. vivax* e bezerros mais soropositivos que vacas para *B. bovis* e *B. bigemina*. Maior número de animais soropositivos para um único agente foi visto para *T. vivax* (15,2%). Vacas demonstraram maior co-soropositividade para *T. vivax* + *A. marginale* (25,5%) e bezerros para *T. vivax* + *B. bovis* + *B. bigemina* + *A. marginale* (57,5%). A alta correlação entre a soropositividade para *A. marginale* x *B. bovis* x *B. bigemina* é provavelmente devida à presença do vetor biológico, o carrapato *Rhipicephalus microplus*, nas fazendas estudadas. As vias de transmissão comuns, mediadas por dípteros hematófagos e fômites, podem explicar a alta co-soropositividade das vacas para *A. marginale* e *T. vivax*. A baixa soropositividade para *C. burnetii* é provavelmente devida ao tipo de sistema de criação empregado (extenso). A soropositividade para *A. phagocytophilum* em apenas um animal sugere a ocorrência de reação sorológica cruzada com outro agente do gênero *Anaplasma*.

Palavras-chave: Tripanossomose, Anaplasmose, Babesiose, Febre Q.

Introduction

The Brazilian Pantanal plays an important role in the national economy due to beef cattle production and is also described as one of the most biologically diverse regions in the world (Myers et al., 2000; Alho, 2005). The human occupation of the Pantanal has introduced exotic animals and plants that changed the ecosystem, thus favoring the dissemination of pathogens among the native fauna (Alho, 2005; Fantin-Cruz, 2008). The relationship between hosts, parasites, and the environment when disharmonious can cause an onset of diseases (Herrera et al., 2007). According to Guimarães et al. (2001), ticks are one of the most important ectoparasites and pathogen vectors for farmed animals in the Pantanal region, keeping a close relationship with the changing environmental conditions.

Moreover, beef cattle production in the Pantanal has been changing, resulting in increasing cattle density and replacement of native vegetation cover with exotic grasses. Consequently, the growing contact between domestic animals and native fauna also increases the exchange of pathogens (Cançado et al., 2008). *Anaplasma marginale*, *Babesia bovis*, *Babesia bigemina*, *Coxiella burnetii*, *Trypanosoma vivax* and *Anaplasma phagocytophilum* infections can cause serious diseases in cattle and are mainly transmitted by arthropod vectors (Maurin & Raoult, 1999; Fereig et al., 2017). Tick-borne diseases are an important factor affecting livestock production in tropical and subtropical regions worldwide, with considerable economic losses (Marcelino et al., 2012).

Anaplasma marginale is an intra-erythrocyte bacterium that has an economic impact on endemic countries, especially due to the high morbidity and mortality in susceptible cattle herds. Losses result from low weight gain, reduced milk yield, abortion, treatment costs and mortality (Kocan et al., 2003). Brazil is endemic for *A. marginale*, with a molecular prevalence in domestic ruminants varying from 7.5% to close to or equal to 100% (Silva et al., 2016). Bovine babesiosis is a hemoparasitosis caused by the protozoa *Babesia bovis* and *Babesia bigemina*, which cause fever, anemia, anorexia, lethargy, hemoglobinuria, and muscle tremors (Singh et al., 2009). This disease is also economically important due to the high morbidity and mortality of animals, especially among calves, and its effects on weight gain and milk yield, requiring expensive control and prevention measures. *Trypanosoma vivax* can cause hematological and nervous changes in cattle, as well as abortions and other reproductive disorders (Silva et al., 1996; Batista et al., 2007).

Coxiella burnetii is an obligate intracellular bacterium that can cause reproductive disorders in ruminants and Q fever in humans. Ruminants are the main source for *C. burnetii*, being responsible for zoonotic outbreaks in several countries (Maurin & Raoult, 1999; Georgiev et al., 2013; OIE, 2018). *Anaplasma phagocytophilum* is a Gram-negative obligate intracellular bacterium that replicates in neutrophilic granulocytes, causing tick-borne fever in sheep and cattle, and granulocytic anaplasmosis in dogs, horses, cats, and humans (Dumler et al., 2001; Carrade et al., 2004; Atif, 2015; Ismail & McBride, 2017; Lappin, 2018; Saleem et al., 2018). The main vectors are ticks of the *Ixodes* complex (*I. ricinus, I. scapularis, I. pacificus, I. persulcatus*, and *I. spinipalpis*) (Stuen et al., 2013).

This work investigated the serological occurrence of *A. marginale*, *B. bovis*. *B. bigemina*, *C. burnetii, A. phagocytophilum* and *T. vivax* in beef cattle in the Brazilian Pantanal.

Material and Methods

Animals and study area

The sampled beef cattle (*Bos taurus indicus*) herds were reared in an extensive farming system in five different farms located in the Central Region of the Pantanal Sul Matogrossense, Sub-Region of Nhecolândia. The blood samples were collected from 400 animals (200 cows and 200 calves) for a cross-sectional study approved by the National Council for Animal Experimentation Control (CONCEA) and the Ethics Committee on Animal Use (CEUA, FCAV, UNESP, Protocol No. 12375/15). All four sampled farms had animals infested with ticks, *Rhipicephalus microplus* and *Amblyomma sculptum,* which were previously identified using already described taxonomic keys (Martins et al., 2010). Some animals presented co-infestation by two or more tick species. Hematophagous dipterans, such as horn flies (*Haematobia irritans*) and tabanids (*Tabanus* spp.) were also observed (Souza Ramos et al., 2019a, b; Mendes et al., 2019; De Mello et al., 2019).

Bovine serum sampling

Whole blood samples collected directly from the caudal vein and stored in 10 mL tubes were centrifuged at 2000 x g for 10 minutes to separate the sera. Serum aliquots were distributed in 2 mL microtubes and stored at -20 ºC until further serological tests.

Indirect enzyme-linked immunosorbent assay (iELISA) for detecting IgG antibodies against *A. marginale, B. bovis, B. bigemina* and *T. vivax*

The antibodies against *B. bovis* and *B. bigemina* were detected by the indirect ELISA technique (iELISA) following the protocol described by Machado et al. (1997), whereas the detection of antibodies to *A. marginale* followed the technique adapted by Andrade et al. (2004). The *A. marginale*, *B. bovis* and *B. bigemina* total antigens were produced by the Immunodot diagnoses Ltda. (Imunodot Desenvolvimento, Indústria e Comércio de Imunógenos e Produtos de Diagnóstico Veterinários, Jaboticabal, SP), at the optimum concentration of 10 μg/mL, diluted in 0.5 M bicarbonate carbonate buffer and pH 9.6. The presence of IgG antibodies against *T. vivax* was evaluated following the modified methodology established by Aquino et al. (1999). The *T. vivax* total antigen was produced in the Laboratory of Immunoparasitology of the Universidade Estadual Paulista (UNESP/FCAV, Jaboticabal, SP) (González et al., 2005) at an optimum concentration of 0.1 μg/mL, also diluted in 0.5M bicarbonate carbonate buffer and pH 9.6. After incubation at 4 ºC for 12 hours, a blockade was performed using PBS Tween 20 (pH 7.2) with added 6% milk powder (Molico®, Nestlé, Brazil). The plates (Maxisorp®; Nunc, Thermo Scientific, Brazil) were incubated in a moist chamber at 37 ºC for 90 minutes. After three washings with PBS-Tween 20 buffer, the positive and negative reference serum and tested serum samples, previously diluted with PBS-Tween 20 solution plus 5% normal Rabbit serum at the following dilutions 1:400 (*A. marginale, B. bovis* and *B. bigemina*) and 1:100 (*T. vivax*)

according to Sampaio et al. (2015), were added to the ELISA plates. The plates were incubated again at 37 °C for 90 minutes. After three washes with PBS-tween 20 buffer, the bovine conjugated IgG (Sigma®, St. Louis, USA) was added to the ELISA plate, in the 1:30000 dilution in PBS-tween 20 plus 5% of normal rabbit serum, with subsequent incubation and washing. Finally, the substrate of the alkaline phosphatase enzyme, P-nitrophenyl phosphate (Sigma®, St. Louis, MO, USA) diluted at 1 mg/mL in diethanolamine buffer pH 9.8 (Sigma®, St. Louis, USA) was added. The iELISA plates were sealed with aluminum foil and incubated at room temperature for 30 minutes for *A. marginale*, *B. bovis* and *B. bigemina* and during 45 minutes for *T. vivax*. The reading was performed in an ELISA reader (B.T.-100; Embrabio, São Paulo, Brazil), with a 405 nm filter. The cutoff values, calculated as 2.5 times the mean absorbance of the negative control sera (Machado et al., 1997), were as follows 0.248 for *B. bovis*; 0.256 for *B. bigemina*; 0.190 for *A. marginale*; and 0.298 for *T. vivax*.

Indirect Fluorescent Antibody Test (IFAT) for detecting IgG antibodies against *C. burnetii* and *A. phagocytophilum*

Detection of IgG antibodies against *C. burnetii* was performed using the crude antigen of the *C. burnetii* At12 strain (Pacheco et al., 2013; Reeves et al., 2006). Serum samples from cattle previously known to be seropositive and/or seronegative for such antigen were used as control (Zanatto et al., 2019a). Detection of IgG antibodies against *A. phagocytophilum* used the crude antigen obtained from HL-60 cells infected with the Webster strain of *A. phagocytophilum*, courtesy of Dr. John Stephen Dumler (Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA) (Scorpio et al., 2004). The positive control sample for *A. phagocytophilum* (1:2560 titer) was obtained from an equine experimentally infected with the Webster strain of *A. phagocytophilum* in the Department of Veterinary Pathology, UNESP, Jaboticabal, SP, Brazil. Serum samples from healthy cattle were used as negative controls (Sousa et al., 2013). Serum samples were first diluted to 1:64 (*C. burnetii*) and 1:80 (*A. phagocytophilum*) in phosphate-buffered saline solution (PBS) (pH 7.2; NaCl 1.3 M, KCl 27 M, Na_2HPO_4 56 mM, KH_2PO_4 10 mM, and NaH_2PO_4 9.2 mM). After dilution, 20 µL and 10 µL serum aliquots were placed in each well of the slides containing antigens against *C. burnetii* and *A. phagocytophilum*, respectively, with posterior incubation in a moist chamber at 37 °C for 30 minutes. After incubation, the slides were washed three times with a wash buffer (phosphate-buffered saline, PBS pH 7.4 + 1% Triton + 1.5 mL of Evans Blue) for five minutes, consecutively, and then dried at room temperature. Then, 20 mL conjugated anti-bovine IgG diluted to 1:200 and marked by Fluorescein isothiocyanate (Sigma-Aldrich®, St. Louis, Missouri, United States) was added to the slides with *C. burnetti* antigens and 10 mL conjugated goat anti-horse IgG diluted to 1:64, also marked with fluorescein isothiocyanate (SIGMA®, St. Louis, Missouri, United States), was added to the slides with *A. phagocytophilum* antigen. The last incubation in a moist chamber at 37 °C for 30 minutes was followed by subsequent washing, as previously described. After the slides were left to dry at room temperature, buffered glycerin was added (glycerin and carbonate-bicarbonate buffer 0.5 M, pH 9.6), the slides were covered with laminules and observed under a microscope equipped with fluorescent light and at 400X magnification (Olympus BX-FLA®, Tokyo, Japan).

Statistical analysis

The correlation between seropositivity for the several investigated agents was estimated by the Pearson Correlation Index (c), using the software The R Project for Statistical Computing (R version 3.4.4). The frequency of co-seropositivity for more than one agent in cows and calves and between male and female calves was analyzed by Chi-Square (x^2) and Fisher exact test. P values < 0.05 were considered statistically significant for one of the categories. In order to check the dependence between seropositive animals and farms, the Chi-square test (x^2) and Fisher's exact test were used, with P <0.05.

Results

The occurrence of antibodies to the selected tick-borne agents and *T. vivax* is shown in Table 1. The Chi-Square (x^2) test showed that *T. vivax* seropositivity was higher in cows compared to calves (P < 0.05) while *B. bovis* and *B. bigemina* seropositivity was higher for calves (P < 0.05). There was no statistical difference (P > 0.05) between the categories for *A. marginale* in the Chi-square test. Furthermore, Fisher's exact test indicated no statistical difference for *C. burnetii* and *A. phagocytophilum* between the two categories, cows and calves. All results are shown in Table 1.

Table 1. Differences in the seropositivity of cows and calves for *T. vivax, A. marginale*, *B. bovis, B. bigemina, C. burnetii* and *A. phagocytophilum,* using the Chi-square test (x^2).

Infectious Agents	Cows	Calves
T. vivax	98.5% (197/200) **A**	83.5% (167/200) **B**
*C. burnetii**	1% (2/200) **A**	0% (0/200) **A**
B. bovis	50% (100/200) **B**	72.5% (145/200) **A**
B. bigemina	34.5% (69/200) **B**	68% (136/200) **A**
A. marginale	77% (154/200) **A**	67.5% (135/200) **A**
*A. phagocytophilum**	0.5% (1/200) **A**	0% (0/200) **A**

Different capital letters overwritten in bold (A and B) in the same line indicate statistical difference regarding seropositivity between cows and calves for a given agent by the Chi-square test (x^2), where (A> B). Equal uppercase letters overwritten in bold (A and B) on the same line indicate no statistical difference. *: Regarding the seropositivity for these agents, Fisher's exact test was used.

The Pearson Index (c) showed no correlation between the seropositivity for *C. burnetii* and *A. phagocytophilum* and the presence of antibodies to the other investigated agents, whereas a moderate correlation was observed for the seropositivity for *A. marginale* x *B. bigemina* x *B. bovis* (Table 2).

Table 2. Correlation (c) of seropositive animals for *T. vivax, A. marginale, B. bovis, B. bigemina, C. burnetii* and *A. phagocytophilum* among the 400 cattle sampled in the Brazilian Pantanal.

	T. vivax	*C. burnetii*	*B. bovis*	*B. bigemina*	*A. marginale*	*A. phagocytophilum*
T. vivax	-	0.01	0.17	0.15	0.21	0.01
C. burnetii		-	-0.01	-0.0	-0.03	-0.00
B. bovis			-	**0.51**	**0.38**	0.03
B. bigemina				-	**0.52**	-0.05
A. marginale					-	0.03
A. phagocytophilum						-

Numbers represent values of "c" according to Pearson's correlation coefficient

Seropositivity for a single agent was higher for *T. vivax* (16%; 32/200 cows and 14.5%; 29/200 calves) while no animal was seropositive for only *B. bovis, C. burnetii* and/or *A. phagocytophilum.* In cows, co-seropositivity was higher for *T. vivax + A. marginale* (25.5%; 51/200; P < 0.05) followed by *T. vivax + B. bovis + B. bigemina + A. marginale* (25%; 50/200). However, in calves, co-seropositivity was higher for the four last agents (57.5%; 115/200;

P < 0.05). Only one cow (0.5%; 1/200) was seropositive for all studied agents (Table 3). Additionally, co-seropositivity for *B. bigemina* was higher for female calves compared to males (P < 0.05).

Table 3. Co-seropositivity for *A. marginale*, *A. phagocytophilum*, *B. bovis*, *B. bigemina*, *C. burnetii* and *T. vivax* among the 400 sampled beef cattle (200 cows and 200 calves) in the Brazilian Pantanal.

Agents	Cows	Calves
Anaplasma marginale (Am)	-	0.5% (1/200)
Anaplasma phagocytophilum (A.p.)	-	-
Babesia bovis (B.bo.)	-	-
Babesia bigemina (B.bi)		1.5% (3/200)
Coxiella burnetii (C.b.)	-	-
Trypanosoma vivax (T.v.)	16% (32/200)	14.5% (29/200)
T.v. + A.m.	25.5% (51/200) A	1.5% (3/200) B
T.v. + B.bo.	4.5% (9/200)	8% (16/200)
T.v. B.bi.	1% (2/200)	0.5% (1/200)
B.bo. + A.m.	0.5% (1/200)	-
T.v. + C.b.	0.5% (1/200)	-
B.bi. + A.m.	-	1.5% (3/200)
T.v. + B.bo. + A.m.	18% (36/200) A	2% (4/200) B
T.v. + B.bi. + A.m.	7% (14/200)	4.5% (9/200)
T.v. + B.bo. + B.bi.	-	3.5% (7/200)
T.v. + B.bo. + B.bi. + A.m	25% (50/200) B	57.5% (115/200) A
T.v + B.bo. + A.m + A.p	0.5% (1/200)	-
T.v. + B.bo. + B.bi. + A.m + C.b.	0.5% (1/200)	-

Chi-square (x^2) and Fisher's exact tests demonstrated the existence of significant dependence between the number of seropositive animals for the studied agents and the farms where beef cattle were sampled. Seropositive calves showed significant dependence on both general and comparative farms (P <0.05). Seropositivity for *A. marginale* was detected in all properties studied, but there was no statistical difference between FPA *vs* FNH; FA *vs* FC; FA *vs* FPA e FPA *vs* FNH. The presence of anti-*B. bovis* and *B. bigemina* antibodies in calves were statistically more frequent and significant in relation to farms in comparison FA *vs* FNH; FA *vs* FPA; FPA *vs* FNH. None of the farms presented seropositive calves for *C. burnetii* and *A. phagocytophilum* (P> 0.05). For *T. vivax*, a higher statistical dependence was found when comparing seropositive calves from farms FA *vs* FC e FA *vs* FPA. These results can be seen in Tables 4 and 5 and Figure 1.

Table 4. Seropositivity of calves for *T. vivax*, *A. marginale*, *B. bovis*, *B. bigemina*, *C. burnetii* and *A. phagocytophilum* and their dependence on the five analyzed farms.
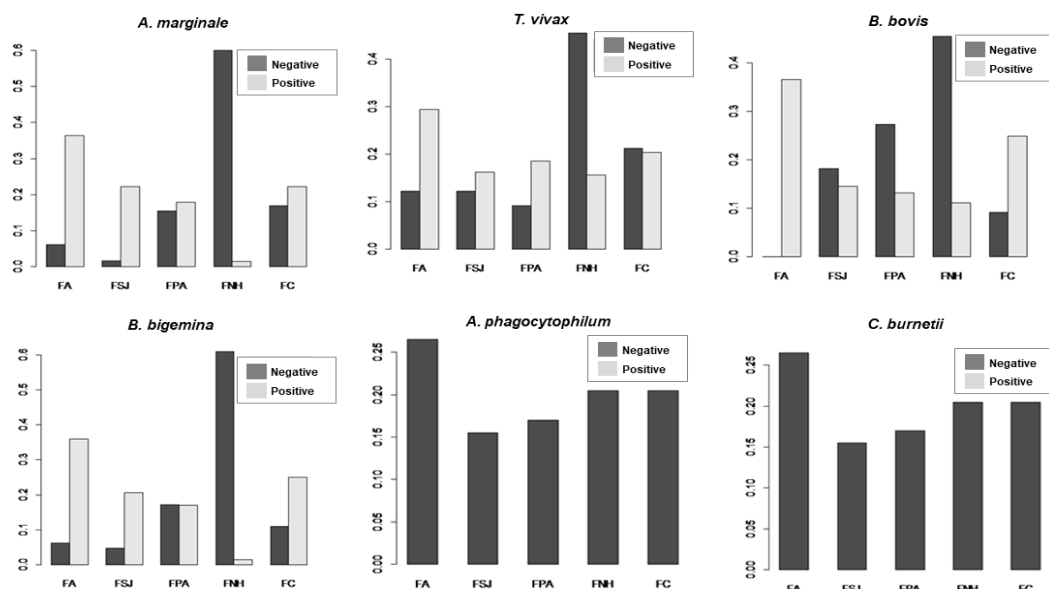
		Farms					
Agents		FA	FC	FNH	FPA	FSJ	*P*-value
A. marginale	POSITIVE	49	30	24	2	30	**< 2.2⁻¹⁶**
	NEGATIVE	4	1	10	39	11	
B. bovis	POSITIVE	53	21	19	16	36	**8.415⁻¹¹**
	NEGATIVE	0	10	15	25	5	
B. bigemina	POSITIVE	49	28	23	2	34	**< 2.2⁻¹⁶**
	NEGATIVE	4	3	11	39	7	
T. vivax	POSITIVE	49	27	31	26	34	**0.002073**
	NEGATIVE	4	4	3	15	7	
C. burnetii	POSITIVE	-	-	-	-	-	0.1257
	NEGATIVE	53	31	34	41	41	
A. phagocytophilum	POSITIVE	-	-	-	-	-	0.1257
	NEGATIVE	53	31	34	41	41	

FA: Alegria Farm; FSJ: São João Farm; FPA: Porto Alegre Farm; FNH: Novo Horizonte Farm; FC: Caceres Farm; *P*-values <0.05 are considered significant by Chi-square (x^2) and Fisher's exact tests.

Table 5. Seropositivity of calves for *T. vivax*, *A. marginale*, *B. bovis*, *B. bigemina*, *C. burnetii* and *A. phagocytophilum* and their dependence on the comparison of the five analyzed farms, represented by *P*-value.

	Farms									
Agents	FA *vs* FC	FA *vs* FNH	FA *vs* FPA	FA *vs* FSJ	FPA *vs* FNH	FSJ *vs* FC	FSJ *vs* FPA	FNH *vs* FC	FNH *vs* FSJ	FC *vs* FPA
A. marginale	**1.606⁻⁰⁵**	**7.051⁻⁰⁸**	**2.516⁻¹²**	**0.01365**	0.1687	**0.04574**	**0.003324**	0.4676	**0.003324**	**0.01898**
T. vivax	**0.01638**	1,0	**0.001292**	0.2158	**0.01138**	0.2386	0.4189	0.2386	1,0	0,2386
B. bovis	0.7054	**0.006979**	**< 2.2⁻¹⁶**	0.201	3.918⁻⁰⁸	0.4987	0.175	0.05497	0.175	2.255⁻¹⁴
B. bigemina	0.4592	**0.006979**	**0.0006655**	0.201	0.01138	0.7472	0.3326	0.7006	0.3326	**0.03139**
C. burnetii	0.1638	0.4165	0.2158	0.2158	0.4189	0.2386	0.4189	0.7098	0.4189	0.2386
A. phagocytophilum	0.1638	0.4165	0.2158	0.2158	0.4189	0.2386	0.4189	0.7098	0.4189	0.2386

FA: Alegria Farm; FSJ: São João Farm; FPA: Porto Alegre Farm; FNH: Novo Horizonte Farm; FC: Caceres Farm; P values <0.05 are considered significant by Chi-square (x^2) and Fisher's exact test.

Figure 1. Seropositive and seronegative calves for the six agents studied according to the farms analyzed. Blank columns represent seropositive animals and black columns seronegative animals.

The seropositivity of cows for *A. marginale, B. bovis* and *B. bigemina* showed significant statistical dependence both in the general comparison of farms and in the comparison of farms to each other (P <0.05). Regarding the seropositivity of cows for *B. bovis*, a higher dependence relationship was observed when the comparisons were performed taking into account two farms at each time. On the other hand, seropositivity for *B. bigemina* only showed significant dependence between FC *vs* FPA farms. There was no statistically significant difference in *T. vivax* seropositivity among the analyzed farms, since the seropositivity for this agent was high in the five sampled farms. A single cow was seropositive for *A. phagocytophilum* on Alegria Farm, whereas two others were seropositive for *C. burnetii* on Porto Alegre and Novo Horizonte farms, respectively. Due to the low number of seropositive animals to *A. phagocytophilum* and *C. burnetii*, there was no significant difference between the analyzed farms (Tables 6 and 7; Figure 2).

Table 6. Seropositivity of calves for *T. vivax*, *A. marginale*, *B. bovis*, *B. bigemina*, *C. burnetii* and *A. phagocytophilum* and their dependence on the five analyzed farms.

Agents		Farms					P-value
		FA	FC	FNH	FPA	FSJ	
A. marginale	POSITIVE	44	32	30	13	35	**3.123⁻¹¹**
	NEGATIVE	1	4	8	26	7	
T. vivax	POSITIVE	45	36	37	37	42	0.1773
	NEGATIVE	0	0	1	2	0	
B. bigemina	POSITIVE	17	25	20	5	2	**4.084⁻¹⁰**
	NEGATIVE	28	11	18	34	40	
B. bovis	POSITIVE	32	20	17	19	12	**0.002018**
	NEGATIVE	13	16	21	20	30	
C. burnetii	POSITIVE	-	-	1	1	-	0.411
	NEGATIVE	45	36	37	38	42	
A. phagocytophilum	POSITIVE	1	-	-	-	-	1.0
	NEGATIVE	44	36	38	39	42	

FA: Alegria Farm; FSJ: São João Farm; FPA: Porto Alegre Farm; FNH: Novo Horizonte Farm; FC: Caceres Farm; P values <0.05 are considered significant by Chi-square (x^2) and Fisher's exact tests.

Table 7. Seropositivity of calves for *T. vivax, A. marginale, B. bovis, B. bigemina, C. burnetii* and *A. phagocytophilum* and their dependence on the comparison of the five analyzed farms, represented by *P*-value.

Agents	FA *vs* FC	FA *vs* FNH	FA *vs* FPA	FA *vs* FSJ	FNH *vs* FPA	FSJ *vs* FC	FSJ *vs* FPA	FC *vs* FPA	FC *vs* FNH	FNH *vs* FSJ
A. marginale	0.1679	0.02448	**0.04529**	**9.956⁻⁵**	0.8205	**0.02131**	0.1652	**0.01898**	0.4446	0.175
T. vivax	1.0	0.4578	0.4643	1.0	1.0	1.0	0.475	0.2386	0.7006	1.0
B. bovis	**0.007014**	0.1915	**0.0126**	**0.000185**	**0.0002325**	**9.348⁻¹⁰**	**1.887e-06**	**2.255⁻¹⁴**	**0.03548**	0.175
B. bigemina	1.0	0.4578	0.2126	1.0	1.0	1.0	0.475	**0.03139**	0.2386	0.3326
C. burnetii	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.2386	0.7098	0.4189
A. phagocytophilum	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

FA: Alegria Farm; FSJ: São João Farm; FPA: Porto Alegre Farm; FNH: Novo Horizonte Farm; FC: Caceres Farm; *P*-values <0.05 are considered significant by Chi-square (x^2) and Fisher's exact test.
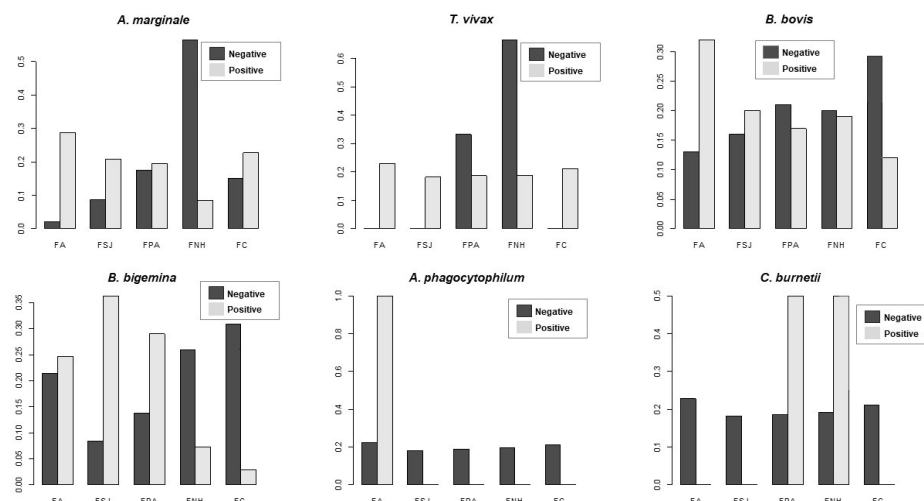
Figure 2. Seropositive and seronegative cows for the six agents studied according to the analyzed farms. Blank columns represent seropositive animals and black columns seronegative animals.

Discussion

In Brazil, the expanding geographic distribution of *T. vivax* has resulted from the livestock moving between endemic regions and those where the conditions are favorable (climate favorable to hematophagous dipterans, animals lacking prior immunity to the parasite, and the permanent practice of sharing blood-contaminated needles [fomites] during vaccination and application of oxytocin) (Cadioli et al., 2012; Bastos et al., 2017; Andrade Neto et al., 2019). The results showed that cows are significantly more exposed to *T. vivax* compared to calves, which may be related to the longer period in contact with hematophagous mechanical vectors and fomites (especially due to shared needles). Hematophagous dipterans (horn and horse flies) were also observed in significant numbers in the sampled properties.

The iELISA results indicated a high occurrence for *A. marginale* in the studied region. In the Brazilian territory, this pathogen occurrence in domestic ruminants estimated by iElisa can range from 16.3% in the semiarid Sergipe to close to 100% in Minas Gerais, Paraíba, Bahia, Paraná, Pará, Rio de Janeiro, São Paulo, and Goiás, being considered an endemic agent in Brazil (Oliveira et al., 2013; Silva et al., 2016). Calves and cows were also exposed to *A. marginale,* due to the constant presence of the tick *Rhipicephalus microplus*, considered a biological vector, and hematophagous dipterans parasitizing both. cows and calves. Additionally, the transplacental transmission may also have contributed to the herd seropositivity, but the occurrence of this transmission mode has not been confirmed among the studied animals. The co-seropositivity for *T. vivax* x *A. marginale* was highest among cows, which remained for a longer period in this breeding system compared to calves and were, therefore, more exposed to the common transmission routes, such as the mechanical vectors and contaminated fomites.

Also, the iELISA results indicated moderate exposure to *B. bovis* (61.25%; 245/400) and *B. bigemina* (51.25%; 205/400) in the sampled beef cattle. Likewise, Barros et al. (2005) reported similar percentages of seropositivity to *B. bovis* (63.7%) in among beef cattle in the municipality of Uauá, Bahia, northeastern Brazil. However, the iELISA seropositivity for *B. bigemina* in the Pantanal region was lower than that found (90.5%; 459/506) among beef cattle in Araguaína, Tocantins (Trindade et al., 2010). According to Mahoney & Ross (1972), endemic stability areas for *B. bovis* and *B. bigemina* comprise those regions where the clinical illness incidence is low, and 75% of calves between 6 and 9 months of age are seropositive for *Babesia* spp. Although clinical cases of bovine babesiosis have not been

diagnosed in the animals sampled in the present study, Pupin et al. (2019) reported 41 clinical cases and anatomopathological lesions of 33 babesiosis outbreaks by *B. bovis* in Zebu cattle of all age groups in the state of Mato Grosso do Sul in the 1995-2017 period.

The Chi-square test showed that calves were significantly (P < 0.05) more seropositive for the studied agents than cows, i.e., the infection frequency was higher for calves. It can be suggested that because the immune system of calves is not yet fully developed, they become, therefore, more susceptible to the infestation by the *B. bovis* vector tick. It is known that Nellore cattle have innate resistance against the fixation of the *R. microplus* tick to the skin, which is avoided by licking (Veríssimo et al., 2015). Thus, it is also hypothesized that calves have lower licking reflex compared to cows, thus allowing a higher number of fixed larvae and nymphs, which would have the sporogenic cycle of *B bovis* and *B. bigemina*, respectively, activated in their salivary glands (Jalovecka et al., 2018). The moderate correlation found among co-seropositive animals for *A. marginale* x *B. bovis* x *B. bigemina,* is probably due to the constant presence of the *R. microplus* tick in the herds, the biological vector common to all three agents. Furthermore, calves had a higher co-seropositivity for *T. vivax* x *A. marginale* x *B. bovis* x *B. bigemina,* which may be related especially to the developing immune system, in addition to contact with common transmission pathways of these agents. The seropositivity of both cows and calves was related to the farm studied, which can be explained by the sanitary conditions of each property, presence of ticks, hematophagous dipterans and reuse of needles.

The beef cattle sampled in the present study showed low exposure to *C. burnetti*. In fact, there are few available data on the serological occurrence of this agent in cattle in Brazil. Recently, Zanatto et al. (2019a) conducted the first study showing *C. burnetii* as a pathogen associated with infectious bovine rhinotracheitis and bovine viral diarrhea, *N. caninum*, *Leptospira* spp., *T. gondii* and *T. vivax* in cattle presenting reproductive disorders in Brazil and reported seropositivity of 13.7% (14/102) among the sampled animals. *Coxiella burnetii* spreads in the herds via aerosol and is commonly related to the extensive farming system (Parker et al., 2006), although ingesting contaminated food and bites of infected ticks are also defined as alternative transmission routes (Eldin et al., 2017). In this sense, the extensive farming system would favor the parasite transmission, mainly via aerosols from urine, feces, and secretions of the infected animals. Considering that the extensive production of livestock predominates in the study region, it is suggested that the concentration of the agent per m^2 is low, decreasing the risk of transmission among the animals.

However, it is important to keep in mind that the Small Cell Variant (SCV), the resistant form of *C. burnetii* can survive for long periods in the environment (Coleman et al., 2004). Zanatto et al. (2019b) demonstrated the first serological evidence of exposure to *C. burnetii* (5.32%, 7/169) of free-living cervids (*Mazama gouazoubira* and *Blastocerus dichotomous*) in Latin America, warning about its importance for an eventual Q fever epidemiology. Interestingly, some of the cervids seropositive for *C. burnetii* were sampled in the Pantanal Brazilian wetland, allowing to raise the possibility that the parasite may be disseminated in the region, despite the low seropositivity found. Furthermore, IFAT detects IgG antibodies against *C. burnetii* in phase I, commonly found during the chronic phase of the disease (Peacock et al., 1983), indicating the possibility that such animals may be infected. Nevertheless, it is not possible to discard the possibility of cross-reaction with other pathogens (Zanatto et al., 2019b).

Also, the IFAT results indicated low seropositivity for *A. phagocytophilum* in the studied cattle. Sacchi et al. (2012) also found seropositivity (16.78%) for *A. phagocytophilum* by IFAT in Pantanal deer (*B. dichotomous*) sampled in the flooded areas of the Paraná River due to the construction of the Porto Primavera hydroelectric plant. However, phylogenetic analysis positioned the found *Anaplasma* sp. genotype close to *Anaplasma platys*. Ebani & Bertelloni (2014) reported that cross-serological reaction with other

species of *Anaplasma* and *Ehrlichia* could generate false-positive results for *A. phagocytophilum* in IFAT. It is known that the *A. phagocytophilum* transmission and distribution are related to the presence of vector ticks of the genus *Ixodes* spp., present only in the USA, Europe, and Asia (Woldehiwet, 2010; Sacchi et al., 2012). The absence of vector ticks in Brazil suggests that seropositivity for *A. phagocytophilum* of a single sample may result from a cross-reaction with another species of *Anaplasma* spp. circulating in cattle in the Brazilian territory. Since cervids and beef cattle under extensive farming system can divide the same grazing area in the Pantanal, it is probable that cattle herds are being exposed to the *Anaplasma* spp. genotype detected in cervids.

Conclusion

The present study revealed that the beef cattle in the Brazilian Pantanal are exposed to tick-borne agents (*A. marginale, B. bovis, B. bigemina, C. burnetii,* and *A. phagocytophilum*) and *T. vivax*. The high seropositivity correlation for *A. marginale* x *B. bovis* x *B. bigemina* is probably due to the presence of the tick biological vector, *R. microplus* in the studied farms. Common transmission pathways, mediated by hematophagous dipterans and fomites, explain the high co-seropositivity of cows for *A. marginale* and *T. vivax*. Low seropositivity to *C. burnetii* is probably due to the type of breeding system employed (extensive). Seropositivity for *A. phagocytophilum* in only one animal suggests the occurrence of a cross-serological reaction with another agent of the genus *Anaplasma*.

Acknowledgements

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