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Prevalence of *Anaplasma marginale*, *Babesia bovis*, and *Babesia bigemina* in cattle in the Campos de Lages region, Santa Catarina state, Brazil, estimated by multiplex-PCR

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ABSTRACT

This study aimed to determine the prevalence of three common hemoparasites (*Anaplasma marginale*, *Babesia bovis* and *Babesia bigemina*) in cattle from 16 counties in the Campos de Lages region, Santa Catarina state, Brazil, and the factors affecting disease occurrence. The study population consisted of 257 clinically healthy animals from 21 rural farms. Bovine blood samples were collected by jugular venipuncture. DNA was extracted from whole blood by the phenol/ chloroform method. Genomic DNA extracted from blood samples was subjected to Multiplex PCR for screening of *B. bovis*, *B. bigemina*, and *A. marginale* using specific primers. Prevalences of *A. marginale*, *B. bigemina*, and *B. bovis* were 27%, 16%, and 29%, respectively. Mixed infection was observed in 17.5% of samples. The most frequent was *Babesia bovis and Babesia bigemina* in 6.62% of samples. *A. marginale* infection rates were statistically correlated with age groups of cattle. The infections detected in the study population were considered to be subclinical, based on the presence pathogen DNA and absence of clinical symptoms. Seasonality of the pathogens resulted in various degrees of infection, related to the age of the animals and the season. The Campos de Lages region is characterized by enzootic instability for these pathogens because of its climatic and geographic features.

1. Introduction

The unicellular parasites *Babesia bovis* and *Babesia bigemina* (order Piroplasmida, family Babesiidae), and the rickettsia *Anaplasma marginale* (order Rickettsiales, family Anaplasmataceae), are transmitted by *Rhipicephalus microplus* ticks, among others (Guglielmone, 1995; Kocan, 1995).

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These tick-borne disease (TBD) agents are widely distributed, have similar clinical signs and laboratory findings, and are major causes of economic losses in the livestock industry, worldwide [3, 4. Inbred strains of domesticated ungulates, particularly cattle (Bos taurus), are often susceptible to infection by single or mixed pathogenic agents, which may be present at any bovine age, in clinical or subclinical forms (Gonçalves, 2000). Annual expenditures in the Brazilian cattle industry are roughly US$ 500 million for basic control and prophylaxis of TBD agents, and US$ 2 billion for dealing with tick/ TBD complexes (Grisi et al., 2002). Economic losses are due primarily to increased mortality, reduced milk production, and poor feed conversion (Dantas-Torres and Otranto, 2016; Dantas-Torres et al., 2016).

Brazil is considered an enzootic country for TBD (Dantas-Torres and Otranto, 2016; Dantas-Torres et al., 2016). However, climatic conditions in some regions do not favor the development of ticks throughout the year, resulting in the existence of specific TBD "enzootic instability" areas. This situation is changing, as studies show that climate variations is every decade causing an expansion of the area of habitat suitability for *R. microplus* to regions previously considered colder in South America where the vector was absent. (Estrada-Pena et al., 2005).

Studies conducted in various regions of Brazil has demonstrated, by serological surveys, the prevalence of *A. marginale*, *B. bovis*, and *B. bigemina* in many states with variable epidemiological features (Souza et al., 2013; da Silva et al., 2015; Santos et al., 2017). In Santa Catarina state, the seroprevalence of *Babesia* sp. was evaluated in a few counties (Dalagnol et al., 1999; Souza et al., 2002).

Polymerase chain reaction (PCR)-based techniques are often utilized in epidemiological surveys of TBD (Martins et al., 2010; Shebish et al., 2012; Zhou et al., 2016) and a modified technique known as multiplex PCR (mPCR) has been particularly successful (Figueroa et al., 1993; Canever et al., 2014; Rodríguez et al., 2015). We used mPCR, to be able in a single reaction to demonstrate the presence of the amplicon related to *A. marginale*, *B. bovis*, and *B. bigemina* infections and the prevalence of these agents in bovine herds in the Campos de Lages region of Santa Catarina state.

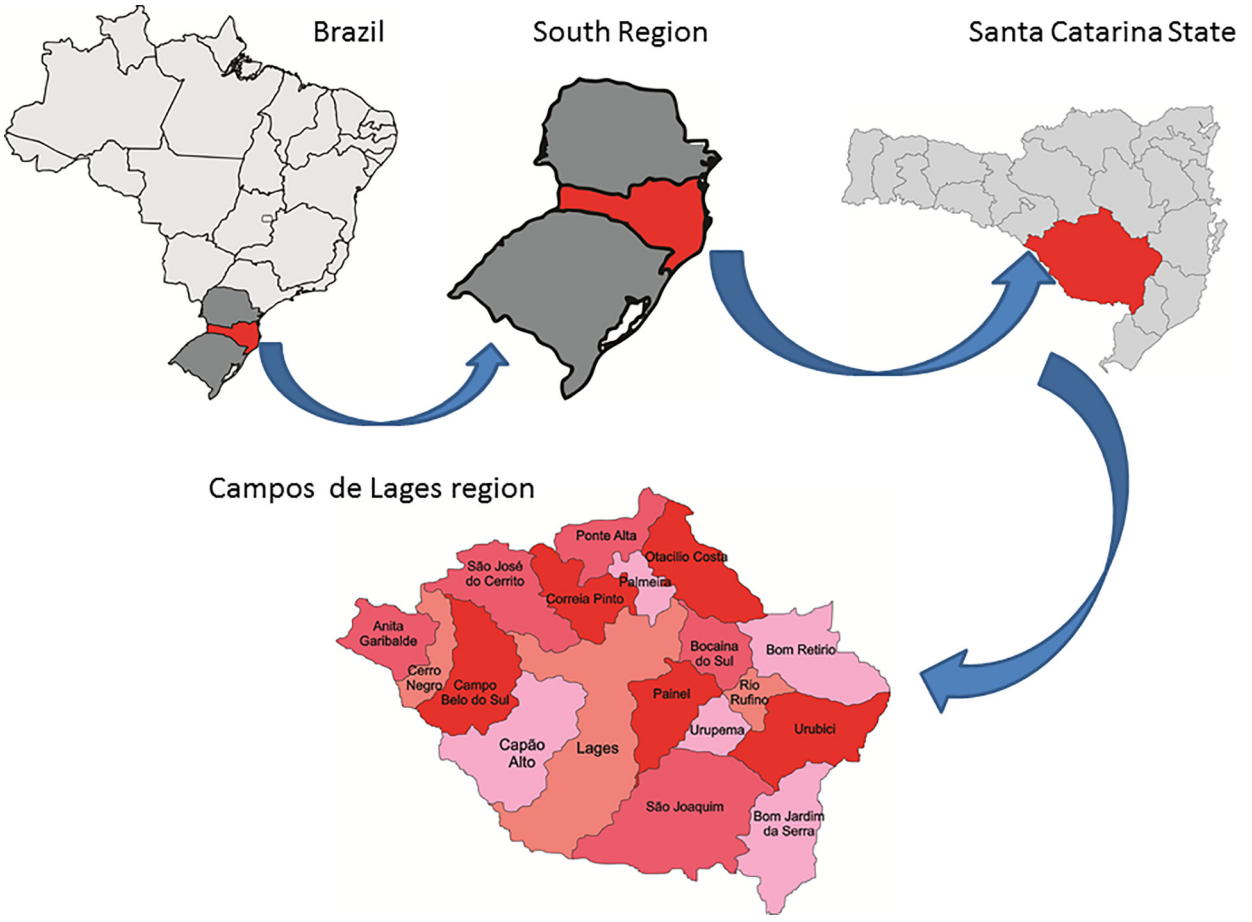

Fig. 1. Geographical location of the Campos de Lages region, Santa Catarina state, Brazil.

2. Materials and methods

2.1. Sample populations

Blood samples were obtained from bovine herds in 16 counties in the Campos de Lages region, Santa Catarina state (Fig. 1), located at altitudes ranging from 850 to 1400 m above sea level. This region is the largest (area 15,726,010 km^2) in Santa Catarina state. Its cities are among the coldest in Brazil, and frequently experience intense frost with occasional snowfall during the winter. Its Köppen climate classification is Cfb (Temperate oceanic climate; coldest month averaging above 0 °C (or −3 all months with average temperatures below 22 °C and at least four months averaging above 10 °C. No significant precipitation difference between seasons) (Peel et al., 2007).

Twenty-one rural farms, having a total of 257 healthy crossbred cattle were selected for sampling in a non-probabilistic manner from March 2012 to June 2013. Determination of sample quantity was based on the equation:

$$n = Z^2 \cdot p \cdot q / E^2$$

where n is sample number, Z is a critical value (confidence level 95%), p is population proportion of positive individuals (admitted 85%), q is the population proportion of individuals who are negativeE is a maximum error of estimation.

Blood samples were collected from 1% or more of animals in each bovine herd.

The properties where the samples were collected had on average 113 animals being, 18 properties of beef cattle and 03 of dairy cattle. Only in two properties there were no reports of previous cases of babesiosis or anaplasmosis.

The bovines were classified into five groups according to age: group 1: ≤6 months; group 2: >6 and ≤ 12 months; group 3: >12 and ≤ 24 months; group 4: >24 and ≤ 36 months; group 5: >36 months. Altitudes of farms were classified into three groups: 1200–1400, 901–1199, and 800–900 m.

2.2. Blood collection

Blood samples were collected by jugular venipuncture using vacuum tubes containing ethylenediaminetetraacetic acid (EDTA). The tubes were placed inside isothermal boxes, and shipped to the Laboratório de Bioquímica de Hemoparasitas e Vetores, Universidade do Estado de Santa Catarina, Lages, Brazil for blood DNA extraction. Ethical and institutional approval was given by the Ethics Committee on the Use of Animals, State University of Santa Catarina Lages-SC, Brazil Number 1.29.11.

2.3. DNA extraction

DNA was extracted from whole blood by the phenol/ chloroform method. In brief, 200 μl whole blood was mixed with lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 25 mM EDTA, 1% SDS) containing 100 μg/ml proteinase K (Sigma; St. Louis, MO, USA), incubated at 42 °C for 12 h, washed sequentially with phenol, phenol/ chloroform (1:1), and chloroform, and centrifuged at 14,000 ×*g* for 10 min. Supernatant was removed, and DNA was precipitated with isopropanol and then washed with 70% ethanol. Tubes were placed in an oven at 37 °C until ethanol was completely evaporated. Resulting DNA was resuspended in 50 μl DNAse-free Milli-Q water, stored at −20 °C, and DNA quantification was measured with a spectrophotometer (NanoDrop 2000H, Thermo Scientific; Waltham, MA, USA).

2.4. Multiplex-PCR

Genomic DNA extracted from blood samples, described as above, was subjected to mPCR amplification for the screening of *B. bovis*, *B. bigemina*, and *A. marginale*.

Table 1
Oligonucleotides primers used in Multiplex-PCR.

Pathogen	Primer	Sequence (5′ -3′)	Amplicon size	Reference
Babesia bigemina	BilA	CATCTAATTTCTCTCCT ACCCCTCC	278pb	(Figueroa et al., 1992)
	BilB	CCTCGGCTTCAACTCTGATGCCAAAG		
Babesia bovis	BoF	CACGAGGAAGGAACTACCGATGTTGA	356pb	(Palmer et al., 1991)
	BoR	CCAAGGAGCTTCAACGTACGAGGTCA		
Anaplasma marginale	1773F	TGTGCTTATGGCAGACATTTCC	1000pb	(Lew et al., 2002)
	2957R	AAACCTTGTAGCCCCAACTTATCC		

The set of primers used for *A. marginale* was described by (Lew et al., 2002), based on the gene encoding Msp1a protein and amplifying 1000 base pairs amplicon. The BoF/BoR and BilA/BilB primers were derived from DNA sequences of *B. bovis* 356-bp (Palmer et al., 1991) and *B. bigemina* 278 bp (Figueroa et al., 1992), respectively, which were used by (Figueroa et al., 1993) in multiplex PCR.

PCR assay was performed in a total volume of 25 μl containing 80 ng genomic DNA and 8.5 pmol of appropriate primer pair (Table 1), added with 1 U *Taq* DNA polymerase, GoTaq® Hot Start Polymerase (Promega Corp.; Madison, WI, USA), 0.2 mM dNTPs, 25 mM $MgCl_2$, 5 μl 5× Green GoTaq® Flexi buffer (Promega Corp.; Madison, WI, USA, and ultrapure water. The reaction was performed in a MaxyGene H Thermal Cycler (Axygen; Union City, CA, USA). Amplification steps were: hot start of 5 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 58 °C, 1 min at 72 °C, and final extension step 10 min at 72 °C. The mPCR sensitivity was based in previous study (Figueroa et al., 1993).

Amplified PCR products were electrophoresed on 0.8% agarose gel, stained with Gel Red dye (Biotium; Fremont, CA, USA), visualized under UV light, and photographed in transilluminator apparatus. Molecular sizes of amplicons were estimated by comparison with a 100-bp ladder standard (Ludwig Biotecnologia; Porto Alegre, Brazil). *A. marginale* (Strain PR1; Londrina), *B. bigemina*, and *B. bovis* strains, kindly provided by Prof. Dr. Odilon Vidotto (State University of Londrina, Brazil), were used as positive controls. Distilled water was used as negative control.

2.5. Sequencing

Purified PCR products were ligated into p-GEM-T-easy® vector (Promega Corp.; Madison, WI, USA) and transformed into calcium-competent `Escherichia coli` cells (DH10BTM, Life Technologies; Grand Island, NY, USA). Single colonies were grown overnight into LB Medium, and plasmids were purified using a plasmid purification kit (QIAGEN; Hilden, Germany). For confirmation of gene insertion, DNA (1–2 μg) was purified from white positive colonies and sequenced by sequencing service facility ACTGene (Porto Alegre, Brazil). High-quality DNA sequences (Phred P20) were analyzed using the Phred/Phrap/Consed software package. Sequence identities were confirmed with the BLAST tool (www.ncbi.nlm.nih.gov/blast).

2.6. Statistical analysis

Logistic regression analysis of a generalized linear model with binomial distribution was performed using the GENMOD procedure of the SAS/STAT statistical software package (https://support.sas.com/rnd/app/stat/procedures/genmod.html). Values of positive or negative were applied to the variables age, season, and altitude.

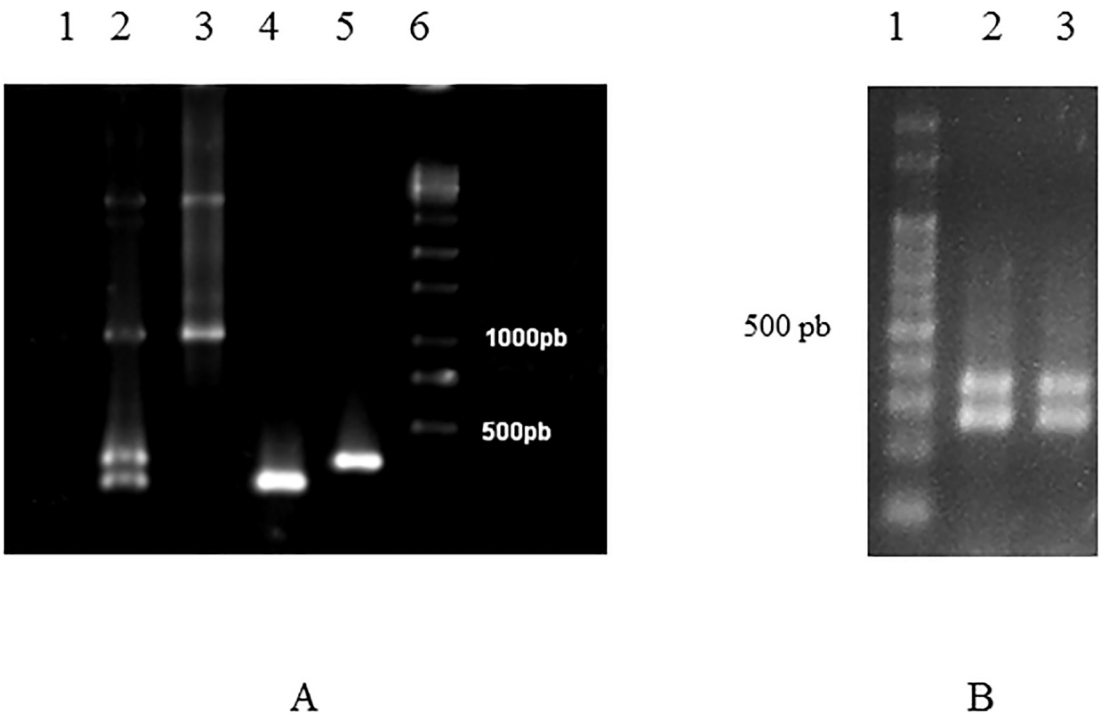

Fig. 2. Multiplex PCR in 0.8% agarose gel. (A) Multiplex PCR and Single PCR for positive controls of *Anaplasma marginale*, *Babesia bovis*, and *Babesia bigemina*, 1: negative control; 2: mPCR; 3: *Anaplasma marginale*; 4: *Babesia bigemina*; 5: *Babesia bovis*; 6: 1000-bp molecular weight marker. (B) Example of samples amplified by Multiplex PCR. 1: 1000-bp molecular weight marker; 2 and 3: *B. bigemina* and *Babesia bovis*.

Table 2
Distribution of individual and concomitant infection in the study population.

Type of infection	Pathogens		Total (%)
Individual	*Anaplasma marginale*	16.34	36.28
	Babesia bovis	14.39	
	Babesia bigemina	5.55	
	Anaplasma marginale and *Babesia bovis*	6.22	
	Anaplasma marginale and *Babesia bigemina*	2.33	
	Anaplasma marginale, *Babesia bovis* and *Babesia bigemina*	2.33	
Coinfection	*Babesia bovis* and *Babesia bigemina*	6.61	17.49

3. Results

Single PCR and mPCR analysis for positive controls indicated 278-bp, 356-bp, and 1000-bp amplicons for *B. bigemina*, *B. bovis*, and *A. marginale*, respectively (Fig. 2).

Of the 257 animals tested, 53.8% were positive for at least one of the pathogens tested. Observed prevalences were 27% for *A. marginale*, 16% for *B. bigemina*, and 29% for *B. bovis*. Total percentages of pathogens were 36.3% for single infections and 17.5% for mixed infections (Table 2).

Infection rates showed significant differences among the five age groups (Table 3). The rate of *A. marginale* infection was lowest in the age 24–36 months group. The rates of *B. bigemina* and *B. bovis* infection did not differ significantly among the age groups.

B. bigemina infection rate differed significantly for winter (3.1%) *vs.* spring, summer, or autumn (>20%) (Table 4). Such seasonal differences for *B. bovis* and *A. marginale* infection rates were not stastically significant. However, for all three pathogens, infection rates were highest in spring.

B. bovis infection rate was significantly higher for the altitude range 901–1199 m (Table 5). *A. marginale* and *B. bigemina* infection rates were also higher for this range, but the differences were not stastically significant.

Distributions of the three pathogens varied among the 16 counties sampled (Table 6). In general, prevalences were highest in São Joaquim, Lages, Painel, and Urupema counties, and lowest in Cerro Negro, Anita Garibaldi, and Ponte Alta counties. Palmeira was the only county in which all the 04 samples collected were negative.

4. Discussion

Molecular tools have become essential during recent decades for epidemiological investigations of parasites. Multiplex-PCR (mPCR), in particular, is useful for simultaneous identification of two or more pathogenic microorganisms and facilitates analysis of large numbers of samples.

Table 3
Percentage of infected animals with different pathogens about animals age.

Age (months)	Animals	*Anaplasma marginale*	*Babesia bovis*	*Babesia bigemina*
	N	%	%	%
≤ 6	27	33.33[a]	29.63[a]	14.81[a]
> 6 ≤ 12	70	28.57[a]	30.00[a]	18.57[a]
> 12 ≤24	71	26.76[a]	35.21[a]	19.72[a]
> 24 ≤ 36	39	7.69[b]	20.51[a]	12.82[a]
> 36	38	31.58[a]	28.95[a]	10.53[a]
P	–	0.0378	0.6082	0.6838

[a,b]Existence of statistical difference.

Table 4
Percentage of infected animals with different pathogens about annual season.

Season	Animals	*Anaplasma marginale*	*Babesia bovis*	*Babesia bigemina*
	N	%	%	%
Summer	90	23.33	23.33	15.56 [a]
Autumm	107	24.30	33.64	20.56 [a]
Winter	32	28,13	28.13	3.13 [b]
Spring	28	50.00	35.71	21.43 [a]
P	–	0.0793	0.0920	0.0404

[a,b]Existence of statistical difference.

Table 5
Percentage of infected animals with different pathogens about farm altitude.

Counties (nt)	Altitude (meters)	Animals	*Anaplasma marginale*	*Babesia bovis*	*Babesia bigemina*
		N	%	%	%
Bom Jardim da Serra, Urupema, São Joaquim (93)	1200 a 1400	48	31.25	22.92 [b]	14.58
Lages, Capão Alto Painel, Campo Belo do sul (82)	901 a 1199	92	31.52	45.65 [a]	23.91
Ponte Alta, Bom Retiro, Otacílio Costa, Correia Pinto, Bocaina do Sul, Palmeira, São José do Cerrito, Rio Rufino, Anita Garibaldi (82)	800 a 900	117	22.22	19.66 [b]	11.97
	P	–	0.0793	<0.0001	0.1143

[a,b]Existence of statistical difference. nt- number of total animals in the county.

The first study in which mPCR was used for simultaneous detection and sensitive analysis of the hemoparasites *B. bigemina*, *B. bovis*, and *A. marginale* in cattle was described by (Figueroa et al., 1993). One pitfall in mPCR is the possibility of false positive identifications resulting from contamination or nonspecific reactions (Bilgiç et al., 2013). Our experimental approach, based on the original method of (Figueroa et al., 1993), optimized identification techniques for the three pathogenic agents through amplification of *A. marginale* using the primers described by (Canever et al., 2014) and that of *B. bovis* using the primers described by (Rodríguez et al., 2015).

In the present work, mPCR results indicated a higher prevalence of *B. bovis* (29.6%) than of *B. bigemina* (16.7%) in a single infection. However, this finding should not necessarily be interpreted as a universal characteristic of the study region. In a study of an outbreak in Ponte Alta County, (Canever et al., 2014) observed high seroprevalences of *B. bigemina* (63.6%) and *A. marginale (*60.6%) infection. In a study of dairy cows in the Northern Plateau of Santa Catarina state, (Souza et al., 2002) observed a higher seroprevalence of *B. bigemina* (84.5%) than of *B. bovis* (76.8%). Dalagnol et al. (Dalagnol et al., 1999), in studies in Lages, Bom Jardim da Serra, Mafra, and Água Doce counties, reported prevalences ranging from 84 to 100% for *B. bovis* and from 95 to 100% for *B. bigemina*. However, (Dalagnol et al., 1999; Souza et al., 2002) reports are based solely on serological studies in Santa Catarina State, and none of the above three studies examined *A. marginale* seroprevalence. Reported infection levels for all three agents are <75%, indicating an area of enzootic instability (Gonçalves, 2000).

Other studies of the variation of pathogen seroprevalence in Brazil (Barros et al., 2005; Guedes Junior et al., 2008; Amorim et al., 2014) indicate the seasonal variation of the microorganisms involved in TBD complexes and the presence of infectious agents in multiple states. Seroprevalence also appears to be closely associated with individual management practices (*e.g.*, intensive tick control) in rural properties, and annual climatic variations, which directly interfere with the development of effective vectors.

In Brazil only few research groups used mPCR for molecular detection of pathogens (Zhou et al., 2016). Studies by other groups have often used PCR or nested PCR (Oliveira-Sequeira et al., 2005). (Brito et al., 2010; Brito et al., 2013) reported high prevalences for *B. bovis* (98.6%), *B. bigemina* (96.4%), and *A. marginale* (95.1%), indicating enzootic stability in Amazonian cattle. In contrast, much lower prevalences were observed for water buffalo (`Bubalus bubalis`) in the same region (*A. marginale* 5.4%, *B. bovis* 15%, *B. bigemina* 16 (Da Silva et al., 2013a; Da Silva et al., 2013b; Da et al., 2014), suggesting that buffalo are more resistant to the vectors (Da Silva et al., 2014).

Table 6
Prevalence of *Anaplasma marginale, Babesia bovis and Babesia bigemina* in the counties.

Counties (nt)		*Ama*		*Bbo*		*Bbi*	
		n	%	n	%	n	%
São Joaquim (47)	Latitude: 28°29′33″S, Longitude: 49° 56′ 1″ W	15	31.9	30	63.8	15	32.9
Painel (23)	Latitude: 27° 55′ 30″ S, Longitude: 50° 6′ 12″ W.	4	17.4	3	13	6	26
Lages(37)	Latitude: −27°.81′ 67″ S, Longitude: 50° 32′ 64″ W	16	43.2	12	32.4	7	18.9
Urupema (30)	Latitude: 28° 17′ 38″ S, Longitude: 49° 55′ 54″ W.	14	46.6	12	40	8	26.6
São José do Cerrito (15)	Latitude: 27° 39′ 45″ S, Longitude: 50° 34′ 48″ W.	5	33.3	2	13.3	0	0
Rio Rufino(10)	Latitude: 27° 51′ 44″ S, Longitude: 49° 46′ 47″ W.	3	30	4	40	1	10
Anita Garibaldi(10)	Latitude: 27° 41′ 9″ S, Longitude: 51° 7′ 50″ W.	1	10	1	1	0	0
Ponte Alta (10)	Latitude: 27.48′ 43″ S, Longitude: 50° 22′ 41″ W	2	20	2	20	3	30
Bocaina do Sul (4)	Latitude: 27° 44′ 40″ S Longitude: 49° 56′ 40″ W	1	25	0	0	2	50
Bom Jardim da Serra (16)	Latitude: 28° 20′ 25″ S, Longitude: 49° 37′ 29″ W.	0	0	0	0	1	6.25
Palmeira (4)	Latitude: 27° 57′ 94″,S, Longitude: 50° 9′ 37″ W	0	0	0	0	0	0
Otacílio Costa (6)	Latitude: 27° 28′ 59″ S Longitude: 50° 07′ 19″ W	0	0	1	16.6	0	0
Correia Pinto (13)	Latitude: 27° 58′ 62″S Longitude: 50° 21′ 55″ W	1	7.7	1	7.7	0	0
Campo Belo (11)	Latitude: 27° 89′ 85 S Longitude: 50° 45′ 26″ Oeste	2	18	2	18	0	0
Capão Alto (11)	Latitude: 27°93′68 S Longitude: 50° 30′ 51″ W	3	27.3	7	63.3	1	9.1
Bom Retiro (10)	Latitude: 27°80′8″ S Longitude: 49° 32′ 1″ W	4	40	1	10	0	0

n- number of positive animals.
nt- number of total animals in the county.

Prevalences of *B. bovis* and *B. bigemina* infection in the northeastern region of Brazil (Köppen climate classification Bsh) were similar to those reported in the southern region, indicating enzootic instability related to dry climate (Amorim et al., 2014). On the other hand, climate in Maranhão state (also in the northeastern region) is more similar to that of the Amazon region, with observed infection prevalences suggesting enzootic stability (Costa et al., 2015). The vectors are not present throughout the year in the region, resulting in later appearance of infection in cattle (Souza et al., 1988). Such seasonality of vectors leads to varying degrees of age-related and season-related infection, as observed. These vectors are able to survive at altitudes >800 m (Baker et al., 1989), explaining the high prevalences of infection observed in cattle at the high-altitude farms we studied.

The cattle in our study population were clinically healthy, with no signs of apathy, jaundice, anemia, or hemoglobinuria, even though 53.3% of them had one or more of the pathogenic agents. Thus, the infections were considered to be subclinical. Some of the cattle were descended from crosses with zebu or European breeds, which are relatively resistant to these vectors, and consequently to clinical manifestation of TBD (Furlong et al., 2004).

We observed mixed infections (two or three agents) in 17.5% of the study animals and single infections in 35.8%. Co-infection of *Theileria annulata* with *A. marginale* and/or *B. bovis* was observed in 22% of cattle in Turkey (Lew et al., 2002), and co-infection of *B. bovis* and *B. bigemina* was observed in 12% of ticks of the species *Rhipicephalus* (formerly *Boophilus*) *annulatus* in Egypt (Adham et al., 2009).

At the time of the research cattle rearing was focused in a subsistence way, prioritizing animals that had both meat and milk aptitude, which leads to a very high racial miscegenation scenario in rural properties, in this region. For this reason, it was not possible to estimate the number of purebred animals for each farm and municipality collected. However, this brings new information since it brings the results of a research conducted with crossbred races in the region.

5. Conclusions

The Campos de Lages region, Santa Catarina, Brazil is characterized by the enzootic instability of the pathogens studied, probably due to climatic changes and the absence of *R .microplus* throughout the year. mPCR was an effective technique for simultaneous analysis of DNA from multiple pathogen species.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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