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Longitudinal evaluation of humoral immune response and merozoite surface antigen diversity in calves naturally infected with *Babesia bovis*, in São Paulo, Brazil

Avaliação longitudinal da resposta imune humoral e diversidade de antígenos de superfície de merozoítos em bezerros naturalmente infectados com *Babesia bovis*, em São Paulo, Brasil

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Abstract

Babesiosis is an economically important infectious disease affecting cattle worldwide. In order to longitudinally evaluate the humoral immune response against *Babesia bovis* and the merozoite surface antigen diversity of *B. bovis* among naturally infected calves in Taiaçu, Brazil, serum and DNA samples from 15 calves were obtained quarterly, from their birth to 12 months of age. Anti-*B. bovis* IgG antibodies were detected by means of the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). The polymerase chain reaction (PCR) was used to investigate the genetic diversity of *B. bovis*, based on the genes that encode merozoite surface antigens (MSA-1, MSA-2b and MSA-2c). The serological results demonstrated that up to six months of age, all the calves developed active immunity against *B. bovis*. Among the 75 DNA samples evaluated, 2, 4 and 5 sequences of the genes *msa-1*, *msa-2b* and *msa-2c* were obtained. The present study demonstrated that the *msa-1* and *msa-2b* genes sequences amplified from blood DNA of calves positive to *B. bovis* from Taiaçu were genetically distinct, and that *msa-2c* was conserved. All animals were serologically positive to ELISA and IFAT, which used full repertoire of parasite antigens in despite of the genetic diversity of MSAs.

Keywords: Bovine babesiosis, MSA, genetic diversity, serology.

Resumo

A babesiose é uma doença infecciosa economicamente importante que afeta o gado bovino em todo o mundo. Para avaliar longitudinalmente a resposta imune humoral contra *B. bovis* e a diversidade genética de antígenos de superfície de merozoítos de *B. bovis*, entre bezerros naturalmente infectados em Taiaçu, Brasil, amostras de soro e DNA de 15 bezerros, foram obtidos trimestralmente, desde o nascimento até aos 12 meses de idade. Os anticorpos IgG para *B. bovis* foram detectados pelos testes de Imunofluorescência Indireta e Ensaio de Imunoadsorção Enzimática Indireto. A Reação em Cadeia da Polimerase foi utilizada para investigar a diversidade genética de *B. bovis*, com base em genes que codificam antígenos de superfície de merozoítos (MSA-1, MSA-2b e MSA-2c). Os resultados da sorologia demonstraram que até seis meses de idade todos os bezerros desenvolveram imunidade ativa contra *B. bovis*. Entre as 75 amostras de DNA avaliadas, foram obtidas 2, 4 e 5 sequências dos genes *msa-1*, *msa-2b* e *msa-2c*. O presente trabalho demonstrou que as sequências dos genes *msa-1* e *msa-2b* amplificadas do DNA do sangue de amostras positivas a *B. bovis* de bezerros de Taiaçu foram geneticamente distintas, e *msa-2c* conservadas. Todos os animais foram soropositivos ao ELISA e ao IFAT, os quais utilizaram o repertório completo de antígenos parasitários, apesar da diversidade genética dos MSAs.

Palavras-chave: Babesiose bovina, MSA, diversidade genética, sorologia.

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Introduction

Bovine babesiosis is an economically important hemoparasitosis affecting cattle worldwide (MTSHALI & MTSHALI, 2013). It is transmitted by ticks of the family Ixodidae, especially those belonging to the subgenus *Rhipicephalus* (*Boophilus*) spp. (SMITH et al., 2000). In Brazil, bovine babesiosis is caused by the apicomplexan intraerythrocytic protozoa parasites *Babesia bovis* and *B. bigemina*, which are agents of huge economic importance in tropical and subtropical regions (JULIANO et al., 2007; SIVAKUMAR et al., 2013). Among the agents causing bovine babesiosis, *B. bovis* is regarded as the most pathogenic species, causing high mortality among susceptible cattle (GAFFAR et al., 2004; BROWN et al., 2006).

Cattle that recover from infection with *Babesia*, either naturally or following chemotherapeutic intervention, generally maintain persistent presence of the parasite, but are protected against the disease if they become reinfected (BOCK et al., 2004).

Previous studies have shown differences between *B. bovis* strains with distinct biological characteristics. In particular, genetic and antigenic differences have been observed among merozoite surface antigens (MSAs) of *B. bovis* isolates in Australia (BERENS et al., 2005), Mexico (BORGONIO et al., 2008), Mongolia (ALTANGEREL et al., 2012), Sri Lanka (SIVAKUMAR et al., 2013), Thailand (SIMKING et al., 2013), Brazil (NAGANO et al., 2013), Israel (MOLAD et al., 2014) and the Philippines (TATTIYAPONG et al., 2014). *B. bovis* merozoites are known to present at least five glycoproteins on their surfaces, which belong to a family of variable merozoite surface antigens (VMSAs) (FLORIN-CHRISTENSEN et al., 2002). It has been proposed that members of the VMSA family play key roles in the initial attachment of merozoites and sporozoites in the erythrocyte host cell during invasion into intermediate hosts (MOSQUEDA et al., 2002; YOKOYAMA et al., 2006).

The VMSA family of *B. bovis* includes the genes *msa-1*, *msa-2a1*, *msa-2a2*, *msa-2b* and *msa-2c* (FLORIN-CHRISTENSEN et al., 2002; SUAREZ et al., 2000). These antigens are highly immunogenic and contain neutralization-sensitive epitopes (HINES et al., 1992). They have therefore been considered to be candidate antigens for developing subunit vaccines against *B. bovis* (HINES et al., 1992; SUAREZ et al., 2000). However, these surface antigens are genetically diverse among the different isolates of *B. bovis*, which results in antigenic differences among distinct *B. bovis* isolates. In Australia, MSA analysis revealed marked differences between vaccine strains and outbreak isolates (BERENS et al., 2005; LEROITH et al., 2005). Although, the study performed by Shkap et al. (1994) demonstrated cross protection elicited by live vaccines despite the presence of high polymorphisms in the *msa-1* and *msa-2* genes. On the other hand, the study done by Wilkowsky et al. (2003) indicate that the *msa-2c* gene product from the Argentine R1A strain is highly antigenic, is expressed on the surface of merozoites, contains widely conserved B-cell epitopes, elicits high levels of IgG2 antibodies in cattle upon immunization, and plays a significant functional role in the process of erythrocyte invasion. Importantly, these findings indicate that MSA-2c is a novel potential vaccine candidate and diagnostic

antigen. Therefore MSAs may be useful as genetic markers, since the genetic diversity among them may reflect differences in the immune response of host animals (BORGONIO et al., 2008; GENIS et al., 2009).

In Brazil, bovine babesiosis is considered to be an endemic disease (SANTOS et al., 2001; FOLLY et al., 2009), and the seroprevalence to *B. bovis* ranges from 56.4 to 97.9% (TRINDADE et al., 2010). The epidemiological status of the disease varies between unstable and stable areas. Some studies have revealed areas of instability, where the serological prevalence is below 75% (BARROS et al., 2005; SOUZA et al., 2013). On the other hand, seroprevalence is above 75% in regions of enzootic stability (FOLLY et al., 2009; TRINDADE et al., 2010; COSTA et al., 2015). However, a study conducted in the state of Mato Grosso do Sul, in central-western Brazil, an area considered to be enzootically stable, showed decreased levels of anti-*B. bovis* colostral antibodies in calves between 3 and 4 months after birth. During this period, clinical babesiosis may occur due to low humoral immune response (MADRUGA et al., 1984).

The losses due to bovine babesiosis associated with *Rhipicephalus* (*Boophilus*) *microplus* may be severe. They have been estimated as nearly 3.5 million dollars per annum, mainly in areas of enzootic instability (GRISI et al., 2014). Although several studies on the prevalence of anti-*B. bovis* antibodies in dairy cattle have been conducted in Brazil, only limited studies assessing the frequency of anti-*B. bovis* antibodies in calves from birth to 12 months of age have been conducted. Moreover, studies regarding the genetic diversity of *B. bovis* in Brazil are quite scarce. In the state of Bahia, in northeastern Brazil, high genetic diversity of *B. bovis* based on MSA-1 sequences has been reported, thus highlighting the importance of conducting extensive studies on this topic before designing immune control strategies in this country (NAGANO et al., 2013).

The present study aimed to investigate the genetic diversity of *B. bovis* and the frequency of antibodies to *B. bovis* in calves from birth to 12 months of age, in a dairy cattle herd located in southeastern Brazil.

Materials and Methods

Study design

A longitudinal study was conducted on a dairy herd in Taiaçu (latitude 21°15'40" S, longitude 48°29'47" W and altitude 735 m), located in the state of São Paulo, in southeastern Brazil. This experimental study covered both seasons of a 12-month period: the rainy season (between October and March, in which the mean temperature and precipitation are 24.4°C and 187 mm, respectively) and the dry season (between April and September, in which the mean temperature and precipitation are 20.9°C and 30 mm, respectively). From June 2013 to June 2014, fifteen female calves (*Bos taurus taurus* x *Bos taurus indicus*) were evaluated every three months, from birth until they reached 12 months of age. In this herd in Taiaçu, the young animals up to 4 months of age were kept in individual stalls, with access to a pasture area of Tifton 85 grass (*Cynodon dactylon*). When the calves reached 5 months of

age, they were transferred to a rotation area comprising *Brachiaria brizantha* pasture. All the animals were dewormed and de-ticked monthly, using levamisole phosphate (10mg/kg, Ripercol®, Fort Dodge, Brazil) and cypermethrin (150mg/kg, Colosso®, Ouro Fino, Brazil), respectively.

The first blood sampling was performed after the calves had ingested the colostrum, i.e. not more than one hour after their birth, followed by sequential sampling at the ages of 3, 6, 9 and 12 months, respectively, totaling 75 blood samples. All the animals had a healthy appearance at the time of sample collection. Approximately 10 mL of blood was collected from the coccygeal or jugular vein of each animal, into two types of Vacutainer tubes: one containing buffered ethylenediaminetetraacetic acid (EDTA) and the other without anticoagulant. The samples were kept at 4°C during transportation to the laboratory. In order to obtain serum samples, blood samples collected without EDTA were incubated at room temperature for 1 h and were then centrifuged at 1000 ×g for 15 minutes. The blood samples with EDTA were stored at -20°C until DNA extraction was performed.

This project was approved by the university's Ethics Committee under the protocol number #017259/14.

Blood smears

Thin blood smears were prepared using peripheral blood. They were air-dried, fixed in methanol, stained with Giemsa and analyzed for the presence of *B. bovis* in the erythrocytes at 100X magnification.

Serological tests

Indirect fluorescent antibody test (IFAT)

The serum samples were subjected to a previously described protocol for anti-*B. bovis* and anti-*B. bigemina* IgG antibody detection (MACHADO et al., 1994; BARCI et al., 1994). The antigenic substrate used in the serological reactions was prepared in accordance with the protocol described by Machado et al. (1994). This consisted of blood smears containing erythrocytes parasitized by *B. bovis* or *B. bigemina* that had been obtained from splenectomized calves experimentally infected by *B. bovis* or *B. bigemina*. The IFAT slides were analyzed under an epifluorescence microscope (Olympus BX60, Tokyo, Japan) and results were scored as positive or negative based upon the fluorescence emission observed on the positive and negative control tests, respectively.

Enzyme-linked immunosorbent assay (ELISA)

The serum samples were also tested for the presence of IgG antibodies against *B. bovis* and *B. bigemina* by means of the indirect enzyme-linked immunosorbent assay (ELISA), using crude soluble antigen, as previously described by Machado et al. (1997). The cutoff value for the optical density (OD) at 405 nm was determined to be two and a half times (2.5X) the mean value in the negative control serum (0.233 OD for *B. bovis* and 0.275 OD

for *B. bigemina*). Absorbance values at or above this value were considered positive (MACHADO et al., 1997).

Conventional polymerase chain reaction (cPCR)

DNA extraction

DNA was extracted from 200µL of each blood sample using the DNeasy® Blood & Tissue Kit (Qiagen®, Valencia, California, USA), in accordance with the manufacturer's instructions. The DNA concentration and absorbance ratio (260/280 nm) were measured using a spectrophotometer (Nanodrop, Thermo Scientific, USA), and the DNA was then stored at -20 °C until use in amplification reactions.

PCR amplification of target *B. bovis* MSAs

Primers previously designed by Tattiyapong et al. (2014) were used in order to amplify *B. bovis* MSA gene fragments (MSA-1, MSA-2b and MSA-2c). For all the MSA genes, a common reverse primer (MSAr) was used based on the conserved nature of the GPI-anchor region of these genes (SIVAKUMAR et al., 2013).

Two forward primers in different PCR sets were used for *msa-1* amplification. One of them (5'-AGTACTTACCTTTTAAATGACAGCCG-3') targeted some of the Australian MSA-1 sequences (DQ028741, DQ028743, DQ028746 and DQ028747), while the other one (5'-ATGGCTACGTTTGCTCTTTTCATTTCAGC-3') targeted the remaining *msa-1* gene sequences retrieved from GenBank.

PCR amplification of the target genes was performed as previously described by Tattiyapong et al. (2014), with minor modifications. Briefly, 5µL of target DNA was used as a template in 25µL reaction mixtures containing 10X PCR buffer, 1.5mM of MgCl₂, 0.8mM of deoxynucleotide triphosphate mixture (Invitrogen, Carlsbad, California, USA), 1.5U of Taq DNA polymerase (Invitrogen, Carlsbad, California, USA), 0.4µM of the forward and reverse primers (Integrated Technologies, Coralville, Iowa, USA) and ultra-pure sterile water (Promega, Madison, Wisconsin, USA). The PCR cycling conditions were modified slightly, as follows: an initial denaturation at 95°C for 5 min was followed by 45 cycles, each consisting of a denaturing step at 95°C for 30 s, an annealing step at 56° for 1 min for MSA-1 and MSA-2b, and at 58.1°C for 1min for MSA-2c, an extension step at 72°C for 2min, and then a final elongation step at 72°C for 5min. A bovine blood sample positive for *B. bovis* (GenBank accession number KU522551) and ultra-pure sterile water (Promega, Madison, Wisconsin, USA) were used as positive and negative amplification controls, respectively.

The PCR products were subjected to agarose gel electrophoresis with ethidium bromide staining and were viewed under UV light (Chemic Doc Imaging System, Bio Rad). All PCR products showing high intensity of bands with expected sizes were purified using Silica Bead DNA Gel Extraction Kit (Fermentas, São Paulo, SP, Brazil). The PCR products were directly purified and subject to DNA sequencing using the same amplification primers as sequencing primers.

Sequence analysis

The genetic diversity of the sequences determined in the present study was analyzed using the DnaSP v5 software (LIBRADO & ROZAS, 2009). The identity values among the nucleotide sequences were assessed by means of the BLASTn tool of the NCBI GenBank database. In addition, the nucleotide sequences of the MSA-1, MSA-2b and MSA-2c gene fragments were converted into amino acid sequences by means of the Expasy-Translate tool. The percentage of similarity between the Taiaçu sequences was calculated using the EMBOSS Needle Pairwise Sequences Alignment software.

Phylogenetic analysis

Three purified amplified DNA fragments from each positive sample were subjected to sequence confirmation in an automatic sequencer (ABI Prism 310 Genetic Analyzer; Applied Biosystems/Perkin Elmer) in both directions by means of Sanger's method (SANGER et al., 1977). The electropherogram quality was analyzed using the Phred Phrap software (EWING et al., 1998). In this, only nucleotide sequences above 400bp in size and Phred quality ≥ 20 were used. Quality scores were assigned to each base call in automated sequencer traces. Additionally, consensus sequences were obtained through analysis on the sense and antisense sequences using the Phred Phrap software. Initially, MSA-1, MSA-2b and MSA-2c sequences were individually aligned with sequences available in GenBank using Clustal/W (THOMPSON et al., 1994). Subsequently, sequences were cut to the same length (the size of the smallest sequence) and finally were manually adjusted in Bioedit v. 7.0.5.3 (HALL, 1999). Phylogenetic analyses based on maximum likelihood criterion (ML) were inferred by means of RAXML-HPC BlackBox 7.6.3 (STAMATAKIS et al., 2008) through the CIPRES Science Gateway (MILLER et al., 2011). The Akaike information criterion available in Mega 5.05 was applied to identify the most appropriate model of nucleotide substitution.

B-cell epitope prediction

The B-cell epitopes in MSA-1, MSA-2b and MSA-2c amino acid sequences were predicted by the method of Kolaskar & Tongaonkar (1990).

Results

Blood smears

Seventy-five blood smears prepared from all the animals surveyed were observed under an optical microscope. None of them was positive for *B. bovis*.

Serological tests

At the second sampling time, three calves (20%) were seropositive for *B. bovis* and nine calves (60%) for *B. bigemina* according to IFAT; however, all the calves presented antibodies levels for *B. bovis*

and *B. bigemina* that were below the ELISA cutoff value at that time. At six months of age, 93.3% and 100% of the calves were seropositive for *B. bovis* and 100% for *B. bigemina* according to IFAT and ELISA, respectively. At 9 and 12 months of age, all the calves (100%) were seropositive for *B. bovis* and *B. bigemina* in both IFAT and ELISA (Table 1). At the time of blood collection, no clinical signs suggestive of babesiosis were seen, although low infestation by *Rhipicephalus (Boophilus) microplus* ticks was observed in all animals during all sampling time.

PCR targeting B. bovis MSAs

Among the seventy-five DNA blood samples from the 15 calves, visible bands for MSA-1 were obtained in nine samples, for MSA-2b in 28 and for MSA-2c in 34, at different sampling times and from different calves. Unfortunately, lower numbers of sequences were obtained for the genes MSA-1 (n = 2), MSA-2c (n = 5) and MSA-2b (n = 4) because of the low intensity of bands, which precluded obtaining sufficient concentration of amplicons for DNA sequencing (Table 2). Blood samples from six calves were positive for all three *msa* genes (*msa-1*, *msa-2b* and *msa-2c*) in the PCR assays and were also seropositive in IFAT and ELISA. For MSA-1, only reactions containing the forward primer directed towards Australian sequences yielded amplified

Table 1. Comparison between IFAT and ELISA for detecting antibodies against *Babesia bovis* and *B. bigemina* in serum samples from 15 calves in Taiaçu, state of São Paulo, Brazil.

Age (Months)	Tests			
	IFAT		ELISA	
	Number of positive to <i>B. bovis</i>	Number of positive to <i>B. bigemina</i>	Number of positive to <i>B. bovis</i>	Number of positive to <i>B. bigemina</i>
0	0	0	0	0
3	3	9	0	0
6	14	15	15	15
9	15	15	15	15
12	15	14	15	14

Total number at each sampling was 15.

Table 2. GenBank accession numbers for the *msa-1*, *msa-2b* and *msa-2c* gene sequences identified in the present study.

Animal type	Animal ID	GenBank accession numbers		
		MSA-1	MSA-2b	MSA-2c
Catlle	43.2		KU522560	
	43.5			KU522563
	44.2	KX420677		
	46.3			KU522565
	47.2		KU522556	
	48.5			KX420672
	50.3			KX420673
	52.2		KX420675	
	52.5			KU522567
	54.3	KX420676		
	57.2		KU522558	
Total		2	4	5

products; the PCR assay using the other forward primer did not produce any visible PCR amplicons. The sizes of the sequences obtained for the genes *MSA-1*, *MSA-2b* and *MSA-2c* were 809-831, 759-827 and 739-770 bp in length, respectively.

The nucleotide diversity per site (Pi), calculated using the DnaSP v5 software, was higher for *MSA-1* (0.39503) and *MSA-2b* (0.10887) than for *MSA-2c* sequences (0.01918). The identity of nucleotide sequences, as assessed using the BLASTn tool of NCBI GenBank, was lower for *MSA-1* (68%) and *MSA-2b* (80-100%) than for *MSA-2c* sequences (97-98%). Pairwise comparison of deduced *MSA-1*, *MSA-2b* and *MSA-2c* amino acid sequences showed that the similarity of *MSA-1* (59.6%) and *MSA-2b* sequences (65.0-98.7%) from Taiaçu was lower than that of *MSA-2c* (85.1-98.6%), respectively (Table 3).

Phylogenetic analyses on the *MSA-1*, *MSA-2b* and *MSA-2c* genes

Three separate phylogenetic trees were constructed using 2, 4 and 5 nucleotide sequences, based on the *MSA-1* (GenBank accession numbers KX420676 and KX420677), *MSA-2b* (GenBank accession numbers KU522556, KU522558, KU522560 and KX420675) and *MSA-2c* sequences (GenBank accession numbers KU522563, KU522565, KU522567, KX420672 and KX420673) obtained in the present study.

The phylogenetic tree based on *B. bovis* *MSA-1* gene sequences clustered the sequences into ten clades as shown in Figure 1. The clade 1 was formed by most of the *B. bovis* *msa-1* gene sequences from Brazil detected in the state of Bahia (NAGANO et al., 2013) and sequences from other countries around the world, namely Thailand, Mongolia Mexico, the United States of America, Ghana and Sri Lanka. The Taiaçu *msa-1* sequences were positioned in two different clades (clades 4 and 9). Clade 4 was formed by two *msa-1* sequences, one from a calf sampled in Taiaçu and another from Vietnam, respectively. Clade 9 was formed by *B. bovis* *msa-1* gene sequences from Vietnam, Australia and a sequence amplified in the present study.

The *msa-2b* gene sequences clustered into nine clades (Figure 2), while the Brazilian *msa-2b* sequences were positioned in two different clades (clades 4 and 7). One sequence (KU522556) amplified in the present study was positioned in clade 4, together with *B. bovis* *msa-2b* gene sequences from Australia, USA, Thailand and Texas. Three nucleotide sequences from Taiaçu (KU522558, KU522560 and KX420675), one from Vietnam, two from Sri Lanka and three from Israel were grouped together into clade 7.

The phylogenetic analysis based on the *msa-2c* gene showed four clades. *B. bovis* sequences from Brazil were positioned in only one clade (clade 4) (Figure 3). Clade 4 grouped together five

Taiaçu DNA sequences (KU522563, KU522565, KU522567, KX420672 and KX420673) determined in this study and another three previously reported sequences from different regions of Brazil: HM352734-Brazilian, from southeastern Brazil; HM352731-Brazilian, from central-western Brazil; and HM352735-Brazilian, from southern Brazil (RAMOS et al., 2012).

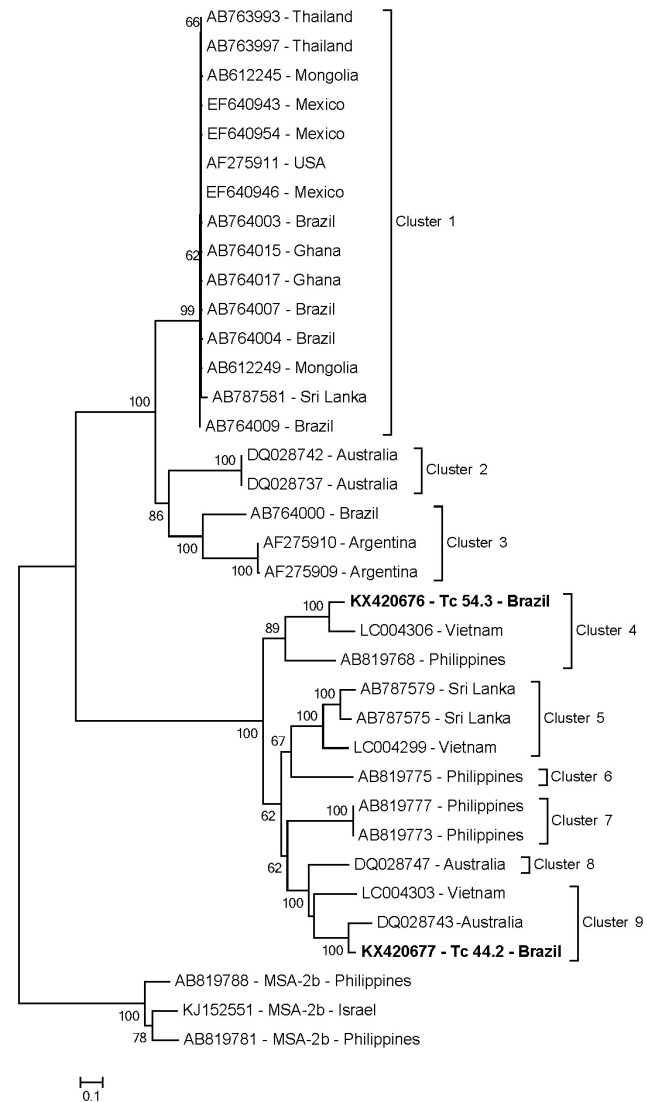


Figure 1. Phylogenetic analyses on *B. bovis* *msa-1* sequences. The sequences determined in the present study are shown in boldface letters. The phylogenetic tree was inferred by using the maximum likelihood method and the GTR+G model. The numbers at the nodes correspond to bootstrap values higher than 60%, accessed with 1.000 replicates. *msa-2b* sequences were used as an outgroup.

Table 3. Nucleotide diversities, identities and similarities of the *msa-1*, *msa-2b*, and *msa-2c* gene sequences from cattle in Taiaçu, state of São Paulo, Brazil.

Gene	Numbers of sequences	Pi ± SD	Identity (%)	Similarity (%)
MSA-1	2	0.39503 ± SD 0.19752	68	59.6
MSA-2b	4	0.10887 ± SD 0.05774	80-100	65.0-98.7
MSA-2c	5	0.01918 ± SD 0.00372	97-98	85.1-98.6

Pi, nucleotide diversity per site; SD, standard deviation.

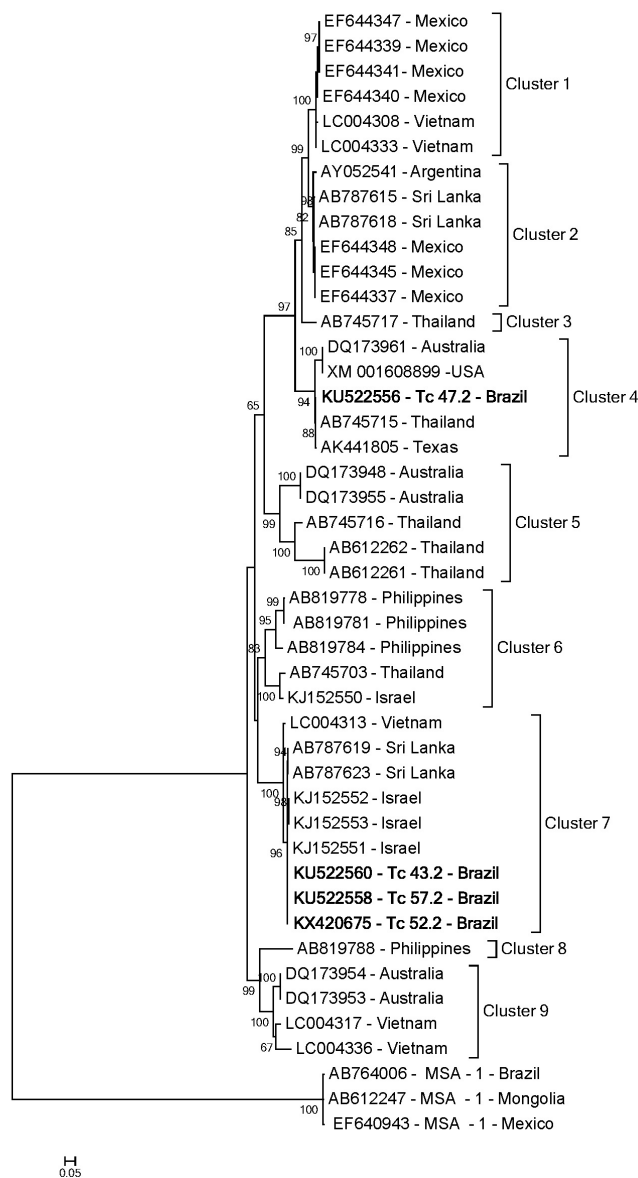


Figure 2. Phylogenetic analyses on *B. bovis* *msa-2b* sequences. The sequences determined in the present study are shown in boldface letters. The phylogenetic tree was inferred by using the maximum likelihood method and the GTR+G+I model. The numbers at the nodes correspond to bootstrap values higher than 70%, accessed with 1.000 replicates. *msa-1* sequences were used as an outgroup.

B-cell epitope prediction

Analyses of Taiacu MSA-1 sequences predicted 11 B-cell epitopes, as shown in Table 4. All epitopes were found to be highly polymorphic, including the epitopes (TSVPESTKKVDDFPDYVPGATT) that were found in the signal peptide region. Interestingly, 6 of such epitopes were observed only in single MSA-1 sequence, indicating that these epitopes were not conserved at all.

Analyses of Taiacu MSA-2b sequences predicted 12 B-cell epitopes, as shown in Table 5. The epitope (KEEDGRYLE) was found in the signal peptide region; and was detected in three of four MSA-2b sequences analysed. In this sequence, all other epitopes

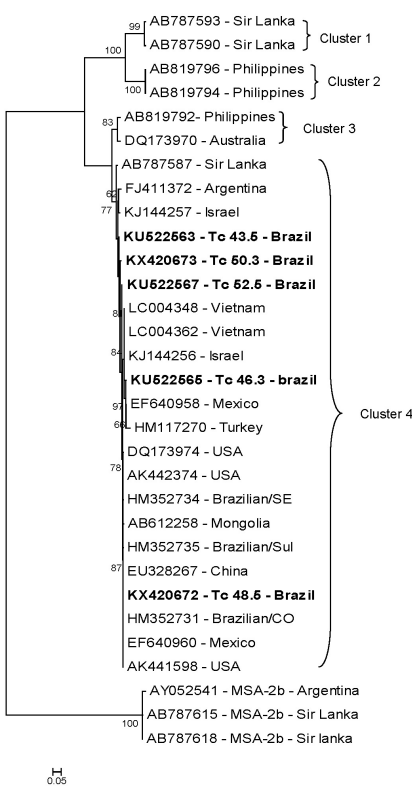


Figure 3. Phylogenetic analyses on *B. bovis* *msa-2c* sequences. The sequences determined in the present study are shown in boldface letters. The phylogenetic tree was inferred by using the maximum likelihood method and the GTR+G model. The numbers at the nodes correspond to bootstrap values higher than 70%, accessed with 1.000 replicates. *msa-2b* sequences were used as an outgroup.

were highly polymorphic with respect to the epitopes detected in the three sequences referenced above. Similar epitopes were observed in three out of four MSA-2b sequences, indicating that these epitopes were conserved in these sequences. Three of such epitopes (HISAKDANVK, IPF and YLDSESPF) (not shown in the table) were observed only in one MSA-2b sequence, indicating that these sequence were not conserved at all. Interestingly, these three nucleotide sequences were positioned into the same clade in the phylogenetic tree, while the single MSA-2b sequence was positioned in a different clade.

Analyses of Taiacu MSA-2c sequences predicted 11 B-cell epitopes, as shown in Table 6. Two epitopes (EAVGMEATSATKTHDAL and RAVPTKQVN) were found in the signal peptide region; the first epitope was detected in four out of five MSA-2c sequences analysed, while the other one was found in one sequence, being observed between the amino acid positions 8 and 16. In general all epitopes detected in this study were found in all five MSA-2c sequences analyzed, but in one sequence those epitopes were found in different amino acid positions in relation to other found sequences. In addition to the epitope in the signal peptide, two other epitopes, namely E and LTKE, were detected in one of MSA-2c sequence in present study, indicating that these epitopes were conserved.

Table 4. Analysis of B-cell epitopes predicted from MSA-1 sequences determined in the present study.

Start position	Stop position	B-cell epitope	Length (aa)	Conserved aa/total aa
11	33	TSVPESTKKVDDFPDYVPGATT	22	2 / 22
35	35	I	1	found in a single sequence
53	68	TLGEELEKVHAGSEQM	16	1 / 16
70-73	77-80	DLVKDAKNGLT	11	2 / 11
87	97	ESIKNDKHTT	11	found in a single sequence
99	114	GFVTAAEDAAAASTNF	16	found in a single sequence
136-139	151-152	DRLFPDNTDNSAKKEEM	17	1 / 17
162	169	NNREVNDNR		found in a single sequence
186	186	D		found in a single sequence
189	192	NAAA		found in a single sequence
182-195	257-270	YQIKSQASGAPSNVDPSSGESSAGTGTTT- SEERDPQPVPAPETASPKEPAPSAQPST- PEGNLHGSQGSTKPTGSSFT	76	12 / 76

After analyzing B-cell epitopes among all MSA-1 sequences, the lengthiest epitopes were selected and used for comparisons.

Table 5. Analysis of B-cell epitopes predicted from MSA-2b sequences determined in the present study.

Start position	Stop position	B-cell epitope	Length (aa)	Conserved aa / total aa
12-13	21	KEEDGRYLE ^a	9	5 / 9
24	36	FKEVNMPSSNSVD ^a	13	6 / 13
51-52	54	KVPF ^a	4	2 / 4
57	59	SLF	3	1 / 3
67	74	LKYQDPDQ ^a	8	4 / 8
85	85	T ^a	1	1 / 1
97-98	115	LNDNPQRLADKNGEVTKY	19	11 / 19
120	130	ICKEDTEVKDY ^a	11	4 / 11
140	143	LDS ^a	3	3 / 3
145	148	SP ^a	2	2 / 2
155	155	N	1	found in a single sequence
159	159	E ^a	1	1 / 1
162	213	KKMPAQSSPTHSSPQVNTTTQPSQD- SAASNTSAGNLNGQQSPNPAGSSFT ^a	71	57 / 71

After analyzing B-cell epitopes among all MSA-2b sequences, the lengthiest epitopes were selected and used for comparisons. ^aThese epitopes were detected in three of four MSA-2b sequences analyzed.

Table 6. Analysis of B-cell epitopes predicted from MSA-2c sequences determined in the present study.

Start position	Stop position	B-cell epitope	Length (aa)	Conserved aa/total aa
8	16	RAVPTKQVN	9	found in a single sequence
25	41	EAVGMEATSATKTHDAL ^a	17	17 / 17
48	61	IKTDAPFNTSDFDT	14	14 / 14
67	73	LSGQSNE ^b	7	7 / 7
94	110	NSFVGESAKHSDKLDTD ^b	17	15 / 17
118	131	NIYDDQSEYNKDKL ^b	14	14 / 14
140	140	T ^c	1	1 / 1
144	146-147	ALRI	4	3 / 4
150	150	E	1	found in a single sequence
152	155	LTKF		found in a single sequence
157	207	TRKAQKDDYRFINPSST- SEAETPSPSSGENTAAQPPK- PAETPKPTGSSFTY	51	50 / 51

After analyzing B-cell epitopes among all MSA-2c sequences, the lengthiest epitopes were selected and used for comparisons. ^aThis epitope was located in the signal peptide in four of five MSA-2c sequences analyzed; ^bThese epitopes were detected in four of five MSA-2c sequences analyzed; ^cThis epitope was detected in three of five MSA-2c sequences.

Discussion

Epidemiological studies on bovine babesiosis in particular areas are important because they may reveal the possibility of occurrences of disease outbreaks. Serological tests are indispensable tools for assessing the immune status and control strategies for *Babesia* spp. infections.

In the present study, blood smears ($n = 75$) were prepared from all the animals surveyed. When these smears were observed under an optical microscope, none of them was positive for *B. bovis*. Although microscopy has been used for the detection of *Babesia* sp. and other hemoparasites, its low sensitivity is a limiting factor in the use of this technique in epidemiological studies of parasites, as in the case of healthy carrier animals (ALMERIA et al., 2001).

Because of the possibility of cross-reactivity between *B. bovis* and *B. bigemina* in this study, serological assays were performed for both species. The results showed that the sampled calves had been exposed to both *Babesia* species. The serological results showed differences in detection of antibodies against *B. bovis* by means of IFAT and ELISA in the samples from the second collection, such that 20% were seropositive according to IFAT and no sample was seropositive according to ELISA. This may have occurred because IFAT detects seropositivity at three months of age, whereas it is not detected by ELISA. On the other hand, in IFAT, the serum is diluted 1:80, while in ELISA, it is at 1:400, which allows detection of serum with low antibody titers by means of IFAT.

The results demonstrated that at six months of age, a time at which the transmission risks are higher, all the calves had already been exposed to *B. bovis* and had thus developed active immunity against this parasite. Additionally, the sampled calves did not present any clinical signs of bovine babesiosis at any time during the experiment. The rearing system used for these calves allowed early contact with *B. bovis*-infected ticks and thus stimulated their active immunity against the parasite. Young calves (less than six months of age) are relatively resistant to developing the severe form of the disease, which is typically observed in susceptible adults upon initial infection with *B. bovis* (TRUEMAN & BLIGHT, 1978; GOFF et al., 2001). However, it is currently accepted that colostral antibodies are not the main source of protection for calves against bovine babesiosis and that innate immune mechanisms are also involved (BOCK et al., 2004). A study conducted in the state of Mato Grosso do Sul, in central-western Brazil, showed decreased levels of anti-*B. bovis* colostral antibodies in calves aged 3-4 months. During this period, clinical babesiosis may occur due to low humoral immune response (MADRUGA et al., 1984).

Exposure of young animals to considerable quantities of *Babesia*-infected ticks during their first nine months of life may ensure natural protection for the rest of their lives, thus resulting in a low risk of clinical disease. Taking into account that the rate of exposure to *B. bovis* in the present work was above 80%, the Taiaçu farm can be characterized as an area of enzootic stability for *B. bovis*. If over 75% of the calves are exposed to *B. bovis*, a given herd is considered to be endemically stable (MAHONEY & ROSS, 1972; TRINDADE et al., 2010; COSTA et al., 2015).

In the present study, the diversity of MSA genes in the *B. bovis* population in cattle in a herd in Taiaçu, southeastern Brazil, was

analyzed. Although a considerable number of MSA gene fragments were obtained, only a few fragments were subjected to sequencing, since most of them yielded bands of weak intensity. The genes encoding MSAs are considered to be useful markers in relation to the molecular epidemiology of *B. bovis*, as confirmed by a number of previous studies (BERENS et al., 2005; LEROITH et al., 2005; ALTANGEREL et al., 2012; SIMKING et al., 2013; SIVAKUMAR et al., 2013; TATTIYAPONG et al., 2014). Genetic diversity is thought to be a survival strategy for protozoan parasites (DEITSCH et al., 2009). Antigenic variation arising from this genetic diversity results in different immune profiles in host animals (BORGONIO et al., 2008; GENIS et al., 2009).

In the phylogenetic analyses, while the Taiaçu *msa-1* and *msa-2b* DNA sequences were detected in multiple clades, the *msa-2c* sequences grouped in only one clade. Few fragments of the *msa-1* gene were amplified, probably because of the presence of polymorphism among the nucleotides in the signal peptide regions of *msa-1* from different *B. bovis* (LEROITH et al., 2006). Additionally, the primer set chosen may have been less efficient than those targeting the *msa-2b* and *msa-2c* genes. Therefore, *msa-1* gene sequences from different *B. bovis* cannot be amplified by a single primer set (NAGANO et al., 2013).

The genetic diversity of MSA-1 and MSA-2b observed in the cladogram was further confirmed by higher values for nucleotide diversity per site (P_i) and low estimated identity and similarity values, and also by the alignments of *msa-1* and *msa-2b* sequences amplified from blood DNA positive to *B. bovis* of calves from Taiaçu (Supplementary figures S1 and S2).

The *msa-2c* gene sequences determined in this study were grouped into only one clade (clade 4). All the *msa-2c* sequences detected in the cattle in Taiaçu grouped together with sequences derived from cattle in several other countries (TATTIYAPONG et al., 2014), including three from other regions of Brazil (RAMOS et al., 2012). This indicates that the *msa-2c* sequences of *B. bovis* shared genetic characteristics worldwide. The fact that *msa-2c* gene DNA sequences from cattle in Taiaçu, Brazil, were positioned in the same clade as other Brazilian DNA sequences may show that there was an absence of genetic diversity. This was supported by low values for nucleotide diversity per site and higher estimated identity and similarity values and sustained by the alignments of *msa-2c* sequences amplified from blood DNA positive to *B. bovis* of calves from Taiaçu (Supplementary figure S3).

A recent study on the *msa-2c* gene reported that the Brazilian *B. bovis* isolates were genetically conserved (RAMOS et al., 2012). Although genetic diversity has been observed in *B. bovis* *msa-1* and *msa-2b* sequences in the present study, a limitation of this work was the small number of sequences obtained for analysis. This was mainly due because PCR products were not amenable for cloning. The cloning approach would not only allow sequencing of the weak amplicons but would also contribute to provide a more diverse pool of sequences present in each single infected animal. In the future, such strategy will contribute to a better clarification of the diversity of *B. bovis* *msa* sequences in Brazil.

In summary, *B. bovis*-VMSA sequence diversity does not seem to correlate with geographic distances.

The ability of native or recombinant-derived MSA-1 to induce merozoite surface reactive antibody that neutralizes merozoite

infectivity (HINES et al., 1992) suggests an important role for MSA-1 in protective immunity against *B. bovis*. In the present study two sequences of *msa-1* gene amplified were positioned in two different clades (clades 4 and 9) and both sequences show more dramatic differences among *msa-1* gene sequences. These differences may be due to the circulation of distinct strains of *B. bovis* or alternatively, due to the selection of “sub-strains” which would be present in a single strain. This selection could have been driven by immune responses in the calves. All these questions can be answered through the development and characterization of biological clones of *B. bovis* which will provide the opportunity to test the role of conserved versus variant antigens in the induction of protective immunity (PALMER et al., 1991; SHKAP et al., 1994).

The use of clones defined by antigenically variant VMSA indicates that although VMSA may contribute to immunity, significant protection against clinical disease is not dependent upon VMSA conservation. Consequently a strategy for vaccine development based on epitopes conserved among otherwise antigenically distinct strains should be followed (SHKAP et al., 1994).

B-cell epitopes can be defined as surface accessible clusters of amino acids, which are recognized by secreted antibodies or B-cell receptors and are able to elicit cellular or humoral immune response (GETZOFF et al., 1988). Identification of epitopes that invoke strong responses from B-cells is one of the key steps in designing effective vaccines against pathogens (POTOCNAKOVA et al., 2016). The presence of polymorphisms in the epitopes might more accurately reflect the functional relevance of the genetic diversity (HINES et al., 1992; SIMKING et al., 2013). In present study, while the predicted epitopes among MSA-1 sequences were poorly conserved, MSA-2b predicted epitopes were relatively conserved; finally, they were conserved among MSA-2c sequences. Many epitopes of MSA-2b and MSA-2c sequences were conserved, it is likely that this peptides functions as an epitope, as they are not located within the signal peptide. The high polymorphisms observed among the epitopes found among Taiaçu MSA-1 sequences may indicate that they differ in their antigenic characters. Since the predicted epitopes among MSA-2c sequences in this present study were conserved; further research will be aimed at determining if such MSA gene is a novel potential vaccine candidate and diagnostic antigen. Although MSA genes have been used as genetic markers, only cross immunity tests could clarify a possible function of this epitopes. (DOMINGUEZ et al., 2010; SIMKING et al., 2013).

Conclusion

The rearing system in which the calves were maintained in the present study allowed exposure of two-month-old animals to *B. bovis*-infected ticks. This favored development of active immunity against the parasite, thus preventing occurrences of clinical signs of the disease.

The *B. bovis msa-1* and *msa-2b* gene sequences detected in these cattle in Taiaçu, southeastern Brazil, were genetically diverse, but the *msa-2c* sequences were conserved.

The serology results showed that calves developed active immunity, which means that they had contact with the agent before the decrease of colostral antibody levels (passive immunity).

The occurrence of antibodies to *B. bovis* crude antigen was not necessarily related to genetic diversity found in *B. bovis-msa* sequences in the present study. Antigenic diverse parasites could evade protective immune responses that may have developed in cattle during past exposure to *B. bovis* parasites. Therefore a study on the genetic diversity of *B. bovis* involving animals from different Brazilian regions is much needed. Additional studies using immunoblot assays and neutralization tests are much needed in order to explore the possibility of using these antigens as potential vaccine components.

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Supplementary Figures

Supplementary material accompanies this paper.

Figure S1. Alignments of *msa-1* sequences amplified from blood DNA positive to *B. bovis* of calves from Taiaçu, State of São Paulo, Brazil

Figure S2. Alignments of *msa-2b* sequences amplified from blood DNA positive to *B. bovis* of calves from Taiaçu, State of São Paulo, Brazil

Figure S3. Alignments of *msa-2c* sequences amplified from blood DNA positive to *B. bovis* of calves from Taiaçu, State of São Paulo, Brazil

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