Original Article

Genetic diversity of *Babesia bovis* studied longitudinally under natural transmission conditions in calves in the state of Rio de Janeiro, Brazil

Diversidade genética de *Babesia bovis* estudada longitudinalmente em condições de transmissão natural entre bezerros no estado do Rio de Janeiro, Brasil

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Abstract

Serum and DNA samples from 15 naturally infected calves in Seropédica, Brazil, were obtained quarterly from birth to 12 months of age, in order to longitudinally evaluate their humoral immune response against *Babesia bovis* and the merozoite surface antigen diversity of *B. bovis*. Anti-*B. bovis* IgG antibodies were detected by an indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). Using DNA amplification, sequencing and phylogenetic analysis, the genetic diversity of *B. bovis* was assessed 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, 0, 3 and 5 sequences of the *msa-1*, *msa-2b* and *msa-2c* genes were obtained, respectively. The present study demonstrated that the *msa-2b* and *msa-2c* gene sequences amplified from blood DNA of *B. bovis*-positive calves were genetically diversified. These data emphasize the importance of conducting deeper studies on the genetic diversity of *B. bovis* in Brazil, in order to design diagnostic antigens and vaccines in the future.

Keywords: Bovine babesiosis, *Babesia bovis*, MSA, genetic diversity, serology.

Resumo

Para avaliar longitudinalmente a resposta imune humoral anti-*B. bovis* e a diversidade genética de antígenos de superfície de merozoítos de *B. bovis,* entre bezerros naturalmente infectados em Seropédica, Brasil, amostras de soro e DNA de 15 bezerros foram obtidas trimestralmente, desde o nascimento até 12 meses de idade. Anticorpos IgG para *B. bovis* foram detectados pelos testes de Imunofluorescência Indireta e Ensaio de Imunoadsorção Enzimático Indireto. Usando-se amplificação de DNA, sequenciamento e análises filogenéticas, a diversidade genética de *B. bovis*, com base nos genes que codificam antígenos de superfície de merozoítos (MSA-1, MSA-2b e MSA-2c) foi investigada. Os resultados da sorologia demonstraram que, até os seis meses de idade, todos os bezerros desenvolveram imunidade ativa contra *B. bovis*. Entre as 75 amostras de DNA avaliadas, foram obtidas 0, 3 e 5 sequências dos genes *msa-1*, *msa-2b* e *msa-2c*. O presente estudo demonstrou que sequências dos genes *msa-2b* e *msa-2c* amplificadas a partir de amostras de sangue positivas para *B. bovis* de bezerros de Seropédica, foram geneticamente distintas. O presente trabalho realça a importância de se realizar estudos aprofundados sobre a diversidade genética de *B. bovis* no Brasil, objetivando o desenvolvimento de antígenos para o diagnóstico e vacinas no futuro.

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

Introduction

Bovine babesiosis is a hemoparasitosis caused by protozoa of the genus *Babesia* and transmitted by *Rhipicephalus microplus* ticks. The disease is mainly characterized by hemolytic anemia and fever, with occasional hemoglobinuria and death (Mosqueda et al., 2012). Bovine babesiosis is an important and widespread cattle disease in tropical and subtropical areas worldwide (Bock et al., 2004). The cattle industry is particularly affected by *Babesia* parasites, since half of the 1.2 billion cattle in the world are at risk of infection and disease (Bock et al., 2004; Grisi et al., 2014).

In Brazil, bovine babesiosis is a serious problem not only in enzootic unstable areas, where most of the calves are not infected with *Babesia* spp. prior to reduction of their maternal antibodies; but also in enzootic stable regions, due to introduction of cattle from tick-free areas (Trindade et al., 2010).

The well-characterized merozoite surface antigen (MSA) family comprises variable proteins that are expressed on the surface of *B. bovis* merozoites and sporozoites. MSAs play a crucial role during invasion of the parasite into host erythrocytes (Mosqueda et al., 2002; Yokoyama et al., 2006). This family includes the *msa-1* gene and *msa-2* loci. While *msa-1* is a single genome copy gene, *msa-2* comprises four tandem genes, namely: *msa-2a1*, *msa-2a2*, *msa-2b* and *msa-2c* (Florin-Christensen et al., 2002). Besides contain neutralizing-sensitive epitopes, these antigens are highly immunogenic and therefore have been considered to be candidates for development of vaccines against *B. bovis* (Mendes et al., 2019). However, the high diversity of these surface antigens is an important obstacle to their use for controlling bovine babesiosis (Genis et al., 2009; Altangerel et al., 2012).

The genetic diversity of *B. bovis* was previously shown based on MSA genes among cattle sampled in Australia (Berens et al., 2005; LeRoith et al., 2005), Brazil (Nagano et al., 2013; Matos et al., 2017; Simking et al., 2013; Mendes et al., 2019), Sri Lanka (Sivakumar et al., 2013), Philippines (Tattiyapong et al., 2014) and Israel (Molad et al., 2014). Antigenic variations arising from this genetic diversity result in different immune profiles in their hosts. Additionally, the involvement of genetic diversity in immune evasion by *B. bovis* is well documented in the literature (Berens et al., 2005; LeRoith et al., 2005).

Only live attenuated vaccines are currently available for prevention of clinical babesiosis caused by *B. bovis* (Shkap et al., 2007). However, clinical babesiosis cases have been increasingly reported among vaccinated cattle due to polymorphism of essential antigenic components of parasites, namely MSAs (Brown et al., 2006).

Few studies regarding the genetic diversity of *B. bovis* have been conducted in Brazil. For instance, high genetic diversity of *B. bovis* based on *msa-1* sequences has been reported among cattle in the state of Bahia, northeastern Brazil. This finding highlights the importance of conducting extensive studies on this subject before designing immune control strategies in this country (Nagano et al., 2013). Recently, Matos et al. (2017) reported that while the *B. bovis msa-1* and *msa-2b* gene sequences amplified from calves' blood samples from Taiaçu, São Paulo, southeastern Brazil, were genetically distinct, *msa-2c* sequences were conserved. Moreover, Mendes et al. (2019) targeted the *msa-2b* and *msa-2c* genes and reported high genetic diversity of *B. bovis* in beef cattle sampled in the Pantanal biome, Mato Grosso do Sul, central-western Brazil.

Considering the scarcity of studies investigating the genetic diversity of *B. bovis* in Brazil, coupled with the fact that *msa* genes may represent genetic markers for studying the heterogeneity of this protozoa, the aims of the present study were (i) to analyze the genetic diversity of *B. bovis* based on MSA (MSA-1, MSA-2b and MSA-2c); and (ii) to detect antibodies against *B. bovis* and *Babesia bigemina* in cattle sampled in a herd in the state of Rio de Janeiro, southeastern Brazil, through a longitudinal study.

Materials and Methods

Study design

A longitudinal study was conducted on a dairy herd at the Seropédica Experimental Station (latitude 22° 48' S; longitude 43° 41' W; and altitude 33 m), which is located in the metropolitan microregion of the city of Rio de Janeiro, southeastern Brazil. This experimental study was performed during two periods: the rainy season (between October and March, in which the mean temperature and precipitation are 26 °C and 1320 mm, respectively) and the dry season (between April and September, in which the mean temperature and precipitation are 23 °C and 170 mm, respectively). During May 2012 and May 2013, fifteen calves (*Bos taurus taurus* x *Bos taurus indicus*) were evaluated every three months from birth until 12 months of age. Calves aged 0 to 2 months were kept in a shed, in individual pens, and received 4 kg of milk per day. They also had access to an area of 0.5 ha of *Brachiaria humidicula* from the

age of 15 days onwards. From 3 to 6 months of age, the calves received 4 kg of milk per day and were kept during the day in an area of 1.5 ha of *Brachiaria decumbens* and were brought in at night to individual pens. Between the ages of 7 and 12 months, the calves were transferred to an area of 3 ha of *B. decumbens* and *Panicum maximum*, where they were kept during the day, and were brought into a collective pen at night. All the animals were dewormed and deticked at monthly intervals using ivermectin (1 mg/50 kg; Merial, Brazil) (Silva et al., 2015).

The first blood sampling was performed after the calves had ingested the colostrum, i.e. not more than one hour after their birth. This was followed by sequential sampling at the ages of 3, 6, 9 and 12 months, thus totaling 75 samples. All the animals were apparently healthy 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 EDTA. The samples were kept at 4 °C during transportation to the laboratory. In order to obtain serum samples, the blood samples collected without EDTA were incubated at room temperature for 1 h and then centrifuged at 1000 ×g for 15 minutes. The EDTA blood samples were store at -20 °C until DNA extraction.

This project was approved by the university's ethics committee, under the protocol number 017259/14.

Serological assays

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 substrates used in the serological assays were prepared in accordance with a previously described protocol (Machado et al., 1994). They 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 results were analyzed under an epifluorescence microscope (Olympus BX60, Tokyo, Japan) with magnification of 40X. Serum samples that were reactive at dilutions ≥ 1:80 were considered positive.

Enzyme-linked immunosorbent assay (ELISA)

The serum samples were also tested for the presence of IgG antibodies against *B. bovis* and *B. bigemina* by an indirect enzyme-linked immunosorbent assay (ELISA), using crude soluble antigens, 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 of the negative control serum samples (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 PCR assays

DNA extraction

DNA was extracted from 200 µL 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). The DNA samples were then stored at -20 °C until their use in PCR assays.

PCR amplification and purification of target *B. bovis msa* gene fragments

Primers previously designed by Tattiyapong et al. (2014) were used in order to amplify *B. bovis msa* gene fragments (*msa-1, ms*a-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.

Two forward primers in different PCR sets were used for *msa-1* amplification. One of them (5-TACTTACCTTTTTAATGACAGCCG-3`) targeted some of the Australian *msa-1* sequences (DQ028741, DQO28743, 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, 5 µL of target DNA was used as a template in 25 µL reaction mixtures containing 10X PCR buffer, 1.5 mM of MgCl$_2$, 0.8 mM of deoxynucleotide triphosphate mixture (Invitrogen, Carlsbad, California, USA), 1.5 U of Taq DNA polymerase (Invitrogen, Carlsbad, California, USA), 0.4 µM of the forward and reverse primers (Integrated Technologies, Coralville, Iowa, USA) and ultrapure sterile water (Promega, Madison, Wisconsin, USA). The PCR cycling conditions were slightly modified, as follows: an initial denaturation step 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 °C for 1 min for *msa-1* and *msa-2b*, or at 58.1 °C for 1 min for *msa-2c*, and an extension step at 72 °C for 2 min; and then there was a final elongation step at 72 °C for 5 min. A bovine blood sample positive for *B. bovis* (GenBank accession number KU522551) and ultrapure sterile water (Promega, Madison, Wisconsin, USA) were used as the positive and negative amplification controls, respectively. The PCR products were subjected to electrophoresis on agarose gel (1%) with ethidium bromide staining and were viewed under UV light (Chemic Doc Imaging System, Bio Rad). All the PCR products showing high intensity of the bands of expected sizes were purified using the Silica Bead DNA gel extraction kit (Fermentas, São Paulo, SP, Brazil).

Phylogenetic analysis

Purified amplified DNA fragments from positive samples were subjected to sequence confirmation in an automated sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems/Perkin Elmer) in both directions, using Sanger's method (Sanger et al., 1977). The electropherogram quality was analyzed using the Phred Phrap software (Ewing et al., 1998), in which only nucleotide sequences above 400 bp in size and Phred quality ≥ 20 were used. Additionally, consensus sequences were obtained through analysis on the sense and antisense sequences using the Phred Phrap software. Initially, *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 down 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 the maximum likelihood (ML) criterion were inferred by means of RAxML-HPC BlackBox 7.6.3 (Stamatakis et al., 2008) through the CIPRES Science Gateway. The Akaike information criterion, available in Mega 5.05, was applied to identify the most appropriate model for nucleotide substitution.

Sequence analysis

The genetic diversity of the sequences obtained in the present study was analyzed using the DnaSP v5 software (Librado & Rozas, 2009). The similarity values among the nucleotide sequences were assessed by BLASTn, available in the NCBI GenBank database. The nucleotide sequences of the *msa-2b* and *msa-2c* gene fragments were converted into amino acid sequences using the Expasy-Translate tool (Artimo et al. 2012). The percentage similarity between the sequences detected in the present study was calculated using the EMBOSS Needle Pairwise Sequences Alignment software (Needleman & Wunsch, 1970).

Results

Serological tests

At the second sampling time, fifteen calves (100%) were seropositive for *B. bovis* and *B. bigemina*, according to IFAT. On the other hand, ten calves (66.66%) were seropositive for *B. bovis* and thirteen (86.66%) for *B. bigemina* according to ELISA. At six months of age, 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 serological tests (Table 1). At the time of blood collection, no clinical signs suggestive of babesiosis were seen.

PCR targeting *B. bovis* MSAs

Among the 75 DNA blood samples analyzed from the 15 calves, there were six, 24 and 34 PCR-positive results regarding the *msa-1*, *msa-2b* and *msa-2c* genes, respectively. However, because of the low intensity of bands (possibly due to low parasitemia during the blood sample collection), which precluded obtaining sufficient concentration of amplicons for cloning and DNA sequencing, only three *msa-2b* and five *msa-2c* sequences were obtained. The sizes of the sequences obtained for the genes *msa-2b* and *msa-2c* were 757-762 and 729-744 bp in length, respectively.

Genetic diversity and phylogenetic analyses on the MSA-2b and MSA-2c genes

The nucleotide diversity per site (Pi), calculated using the DnaSP v5 software, was higher for the *msa-2b* sequences (0.1439 – SD = 0.06) than for the *msa-2c* sequences (0.0600 – SD = 0.01). Pairwise comparison of the deduced MSA-2b and MSA-2c amino acid sequences showed that the degree of similarity of the MSA-2b sequences (61.7-94.2%) was lower than that observed for the MSA-2c sequences (84.3-99.1%) (Table 2). Likewise, the degree of similarity of the MSA-2b sequences (72.6-96.5%) was lower than that of the MSA-2c sequences (89.2-99.5%) (Table 3). Also, the alignment of the MSA-2c amino acid sequences showed that they were distinct from each other (Figure 1).

Two separate phylogenetic trees were constructed using three and five nucleotide sequences, based on the *msa-2b* (GenBank accession numbers KX160813, KX160814 and KX160815) and *msa-2c* (GenBank accession numbers KX160804, KX463632, KX463633, KX463634 and KX463635) sequences, respectively.

The *msa-2b* sequences analyzed in the present study clustered into six clades (Figure 2), while the Brazilian *msa-2b* sequences were positioned in three different clades (clades 1, 3 and 6). Two *msa-2b* sequences amplified in the present study (KX160813, KX160814) were positioned in clade 1, and were grouped with sequences previously reported from Sri Lanka, Thailand, Vietnam, Philippines and Argentina. One *msa-2b* sequence (KU522556) from Taiaçu, São Paulo, was positioned in clade 3 and clustered with sequences from USA and Thailand. Lastly, and

Table 1. Comparison between IFA and ELISA tests in detecting IgG antibodies against *Babesia bovis* and *B. bigemina* in 15 calves' serum samples from a herd in Seropédica, State of São Paulo, Brazil.

Age (Months)	Assays			
	IFAT		ELISA	
	N. of (+) to *B. bovis*	N. of (+) to *B. bigemina*	N. of (+) to *B. bovis*	N. of (+) to *B. bigemina*
0	0	0	0	0
3	15	15	10	13
6	15	15	15	15
9	15	15	15	15
12	15	15	15	15

Total number at each sampling was 15; (+) = positive. N = Number.

Table 2. Percentages of identity among *B. bovis msa-2c* nucleotide sequences from calves sampled in a herd in Seropédica, state of Rio de Janeiro, Brazil.

Sequences	(1)	(2)	(3)	(4)	(5)
KX160804 **(1)**	100%	89.7%	84.3%	89.7%	89.2%
KX463632 **(2)**	89.7%	100%	89.9%	99.1%	90.8%
KX463633 **(3)**	84.3%	89.9%	100%	90.7%	86.2%
KX463634 **(4)**	89.7%	99.1%	90.7%	100%	90.8%
KX463635 **(5)**	89.2%	90.8%	86.2%	90.8%	100%

Table 3. Percentages of similarity among *B. bovis msa-2c* amino acid sequences from calves sampled in a herd in Seropédica, state of Rio de Janeiro, Brazil.

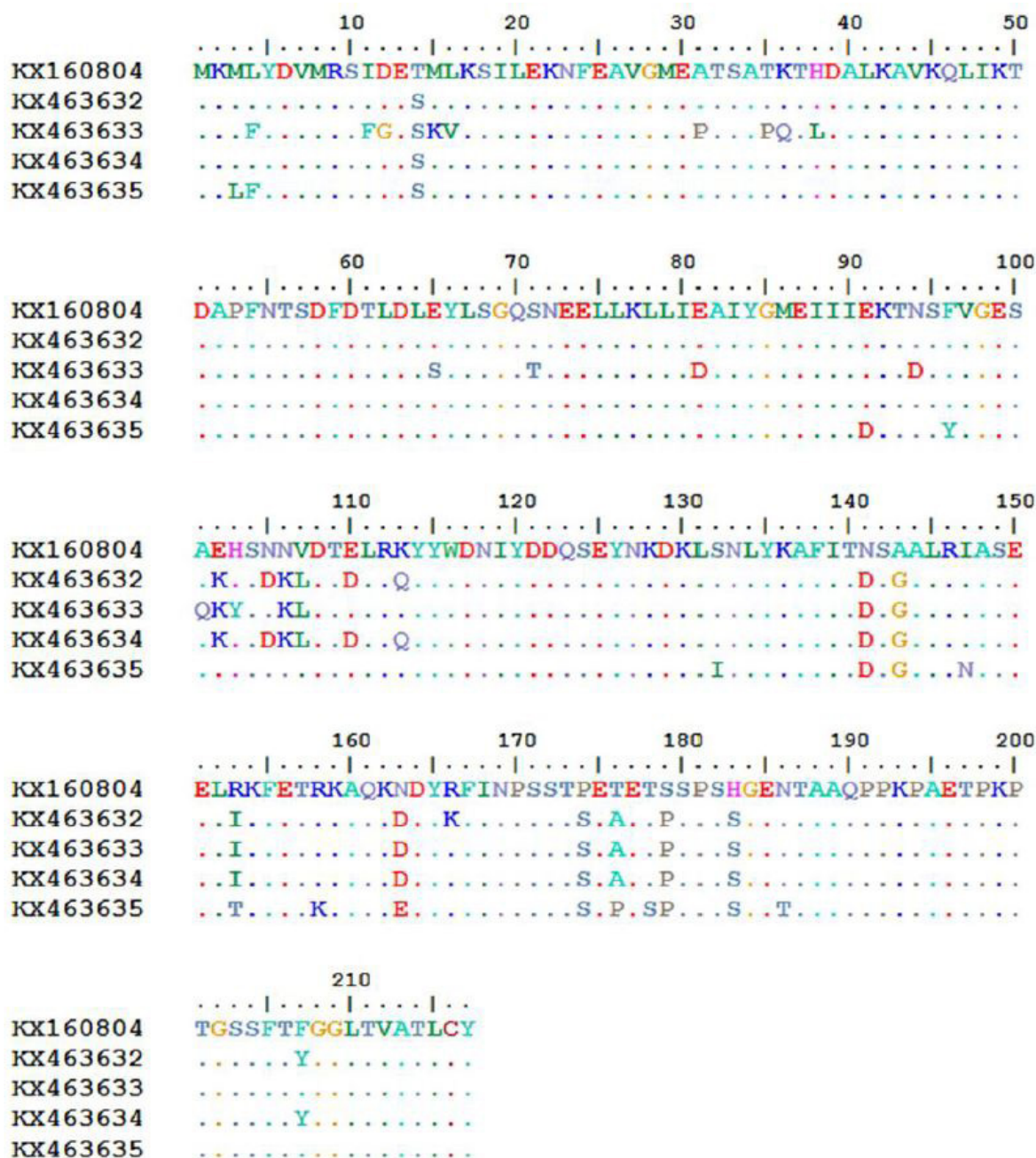| Sequences	(1)	(2)	(3)	(4)	(5)
KX160804 **(1)**	100%	94.2%	89.2%	93.7%	92.4%
KX463632 **(2)**	94.2%	100%	94.9%	99.5	96.8
KX463633 **(3)**	892%	94.9%	100%	95.4	92.6%
KX463634 **(4)**	93.7%	99.5%	95.4%	100%	96.3%
KX463635 **(5)**	92.4%	96.8%	92.6%	96.3	100%

Figure 1. Alignment of the MSA-2c amino acid sequences. The nucleotide sequences of the *msa-2c* gene fragments were converted into amino acid sequences using the Expasy-Translate tool. Thereafter, the amino acid sequences were aligned and compared to each other using the Bioedit.

constituting the clade 6, the other sequence detected in the cattle from Seropédica (KX160815) clustered with four sequences (KU522558, KU522560, KU522562 and KX420675) from Taiaçu (São Paulo, Brazil), Vietnam, Israel and Sir Lanka. All three clades were supported by high bootstrap values (96, 100 and 100%, respectively).

The phylogenetic analysis based on the *msa-2c* gene showed four clades. *Babesia bovis* sequences from Brazil were positioned in two clades (clades 3 and 4) (Figure 3). Only one sequence from Seropédica (KX463633) was positioned in clade 3. Clade 4 grouped four sequences from Seropédica, five from Taiaçu (KU522563, KU522565, KU522567, KX420672 and KX420673), and another three sequences previously reported 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), along with several other sequences previously reported from around the world (Figure 3).
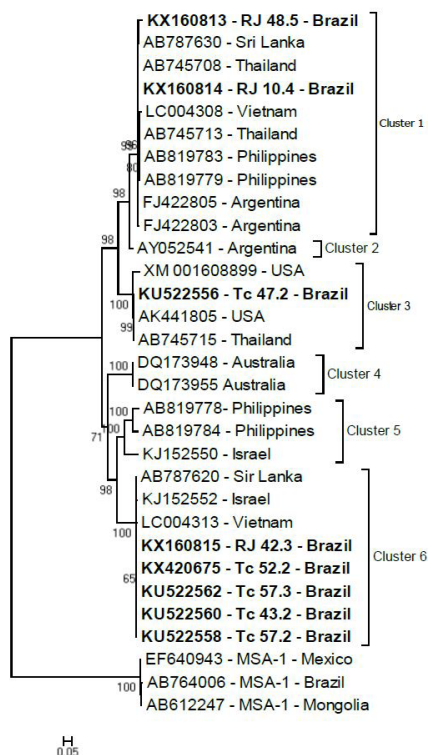
Figure 2. Phylogenetic analyses of *B. bovis msa- 2b* sequences. The sequences determined in the present study are shown in boldface letters and followed by the letters RJ and the sequences from Taiaçu, SP followed by the letters Tc. The Phylogenetic tree was inferred by using the maximum likelihood method and Model GTR+G+I. The numbers at the nodes correspond to boostrap values higher than 65% accessed with 1.000 replicates. *B. bovis* MSA-1 sequences were used as an outgroup.
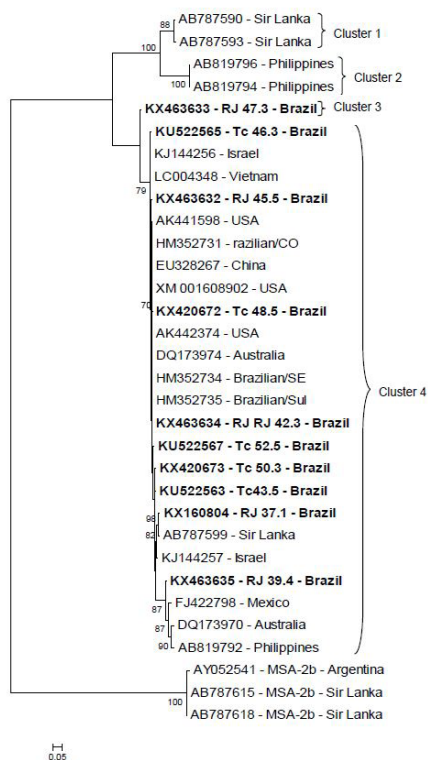

Figure 3. Phylogenetic analyses of *B. bovis msa- 2c* sequences. The sequences determined in the present study are shown in boldface letters and followed by the letters RJ, and the sequences from Taiaçu, SP followed by the letters Tc. The Phylogenetic tree was inferred by using the maximum likelihood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

Discussion

Using serological and molecular approaches, the current study assessed natural exposure to bovine babesiosis and the genetic diversity of *B. bovis* in a cattle herd in Seropédica, Rio de Janeiro.

Serological tests (IFAT and ELISA) have been used as indispensable tools for assessing *B. bovis* epidemiological status in cattle herds in Brazil. Because cross-serological reactivity can occur between *B. bovis* and *B. bigemina,* ELISA tests were performed in order to assess exposure to both *Babesia* species in the present study. The results showed that the calves sampled had been exposed to both *Babesia* species during their first 12 months of life. In fact, at six months of age, all the calves sampled had already been exposed to *B. bovis* and had developed active immunity against this parasite. The rearing system within which these calves were maintained allowed early contact with *B. bovis*-infected ticks, thus stimulating active immunity against the parasite.

Furthermore, it is now 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 carried out in the state of Mato Grosso do Sul, 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).

Given that the rate of exposure to *B. bovis* in the present study was above 80%, the herd analyzed here could be characterized as presenting 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* gene fragments in the *B. bovis* population in cattle in a herd in Seropédica, southeastern Brazil, was analyzed. Although a considerable number of DNA samples were positive, only a few fragments were subjected to sequencing, since most of them yielded faint bands. One possible explanation for these findings may be that the parasitemia levels were low during the sampling. Since our main objective was to evaluate the genetic diversity of *B. bovis* in the same positive animals at different sampling times, the weak intensity of the bands precluded assessment of whether these *msa* gene fragments had become modified through *B. bovis* infection.

The genetic diversity of *msa (msa-1, msa-2b, and msa-2c)* has been analyzed in several *B. bovis*-endemic countries in the recent past (Genis et al., 2009; Altangerel et al., 2012; Sivakumar et al., 2013). Studies conducted in Australia (Berens et al., 2005; LeRoith et al., 2005), Sri Lanka (Sivakumar et al., 2013), the Philippines (Tattiyapong et al., 2014), Thailand (Simking et al., 2013), Israel (Molad et al., 2014), Mexico (Borgonio et al., 2008; Genis et al., 2009) and Brazil (Nagano et al., 2013; Matos et al., 2017; Mendes et al., 2019) have found that *B. bovis* isolates in these countries are genetically diverse, according to their *msa* gene sequences.

Herein, the *B. bovis* genetic diversity (pi: *msa-2b* = 0.1439 and *msa-2c* = 0.0600) assessed using DnaSP was similar to what had previously been reported (pi: *msa-2b* = 0.1735 and *msa-2c* = 0.0322) from beef cattle sampled in the Brazilian Pantanal biome (Mendes et al., 2019).

In the phylogenetic analyses, the Seropédica *msa-2b* and *msa-2c* DNA sequences were positioned into multiple clades. However, it needs to be emphasized that only one *msa-2c* sequence detected was positioned in a separate cluster. Moreover, although the majority of the *msa-2c* sequences were grouped in the same cluster, the alignment and the similarity analysis showed that these sequences were distinct. On the other hand, while the *B. bovis msa-2b* sequences recently obtained from calves in Taiaçu (São Paulo, southeastern Brazil) were detected in multiple clades, *msa-2c* sequences were positioned in a single clade (Matos et al., 2017).

In a previous study targeting the *msa-2c* gene, it was reported that the Brazilian *B. bovis* isolates were genetically conserved (Ramos et al., 2012). However, despite the low number of msa-2c sequences amplified, the current study showed that these sequences were distinct from each other. These finding are in agreement with a previously published study that reported high genetic diversity for this genic locus in the *B. bovis* strains detected in beef cattle in the state of Mato Grosso do Sul, Brazil (Mendes et al., 2019). Additionally, similar results have been described in cattle from Australia (Berens et al., 2005), the Philippines (Tattiyapong et al., 2014) and Israel (Molad et al., 2014). These results suggest that the genetic diversity of *B. bovis* strains is different at distinct sampling points.

The analyses on *msa-2b* gene sequences detected in cattle in Seropédica (RJ) and Taiaçu (SP) (Matos et al., 2017) showed that the *B. bovis* isolates from both localities were positioned into two main genotypic groups. One genotype occurred on both farms. In the same way as in previous studies (Matos et al., 2017; Mendes et al., 2019), the phylogenetic and similarity analyses showed that this fragment was more heterogenic than the *msa-2c* gene fragments.

One limitation of the present study was the small number of sequences obtained for diversity analysis. This was mainly because the PCR products were not amenable for cloning. The cloning approach would contribute towards providing a more diverse pool of sequences present in each infected animal. In the future, the use of this strategy will enable better clarification of the diversity of *B. bovis msa* sequences in Brazil.

In summary, the genetic heterogeneity verified herein may result in antigenic variation in *B. bovis* strains, thus limiting the use of these antigens as recombinant vaccines for use in controlling bovine babesiosis. In addition, the data reported in this study indicate that all the animals turned out to be serologically positive for *B. bovis* during their first 12 months age, although the genetic diversity of MSAs was found not to be conserved. These data emphasize the importance of conducting deeper studies on the genetic diversity of *B. bovis* in Brazil, in order to design diagnostic antigens and vaccines in the future.

Acknowledgements

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