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Original article

Assessment of equine piroplasmids in the Nhecolândia sub-region of Brazilian Pantanal wetland using serological, parasitological, molecular, and hematological approaches

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ABSTRACT

Brazilian Pantanal is the world´s largest wetland ecosystem, where cattle's ranching is the most important economic activity. The objective of this study was to compile some epidemiological features on equine piroplasmids from the Nhecolândia sub-region of Pantanal wetland through the evaluation of the patterns of *T. equi* and *B. caballi* infections in different groups of horses; identification of the tick species that infest horses; and to study phylogenetic relationships among *Theileria equi* 18S rRNA gene sequences. During October 2015, blood and serum samples were collected from 170 horses in four different categories. Ticks, after identification, had their hemolymph and eggs examined for the presence of piroplasmid sporokinets. Also we searched parasites in the peripheral blood smears of the investigated horses. The number of red blood cells (RBCs) and the packed cell volume (PCV) were determined to test for anemia in the infected animals, and exposure to *B. caballi* and *T. equi* was evaluated by enzyme-linked immunosorbent assay. "Catch all primers" based on 18S rRNA gene were used in polymerase chain reactions (PCR) to detect equine piroplasmids, followed by three nested PCRs for the phylogenetic analysis. The serological results showed that 61.8% and 52.9% of the horses sampled were exposed to *T. equi* and *B. caballi*, respectively. Piroplasmid DNA was detected in 43.5% of the horses analyzed. Our sequencing revealed 98–100% identity with some sequences previously published in GenBank for *T. equi*, and microheterogeneity among others. We found that 51.2% of the animals sampled were infested with *Dermacentor nitens*, *Amblyomma sculptum*, and *Rhipicephalus* (*Boophilus*) *microplus,* singly or co-infested. Since positive and negative animals presented similar RBC and PCV values, and no sporokinets were found on blood smears, hemolymph and eggs of the ticks collected, we suggest that infected equines can act as asymptomatic carriers for piroplasmosis in the studied region. Our results together showed the enzootic characteristic of equine piroplasmids in Pantanal region highlighting the importance of using different methods for detection these parasites. Moreover, breeding mares and foals should be monitored since they displayed the greatest occurrences for molecular test (59.0% and 86.1% respectively) and tick infestations (87.2% and 63.9% respectively).

1. Introduction

The order Piroplasmorida includes two families that belong to the phylum Apicomplexa, namely Babesiidae and Theileriidae (Laveran, 1901; Levine, 1988), which contain parasites of great medical, veterinary, and economic importance (Friedhoff et al., 1990; Zobba et al., 2008). They are intracellular parasites of the blood cells vectored by ticks from the family Ixodidae in which sexual reproduction occurs. Piroplasmids that infect equids, *Babesia caballi* and *Theileria equi*, along with their ixodida vectors, have a worldwide distribution (Wilkinson,

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1967; Scoles and Ueti, 2015).

The taxonomy of *T. equi* is controversial. Originally described as *Babesia equi*, it was renamed due to the parasite´s early pre-erythrocytic life cycle in leukocytes. Recently, it was proposed to include *T. equi* in a genus other than *Theileria* and *Babesia*, close to *Cytauxzoon felis*, due to their biological and molecular characteristics (Ramsay et al., 2013; Schreeg et al., 2016).

Equine piroplasmosis is responsible for hemolytic disease, which is characterized by intermittent fever, anemia, jaundice, hemoglobinuria, bilirubinuria, hepatitis, splenomegaly, and eventually death (Holbrook, 1969; de Waal, 1992; Zobba et al., 2008). *Theileria equi* is considered more virulent than *B. caballi*, because it infects leukocytes before erythrocytes during the acute phase, and the infection is persistent (Ramsay et al., 2013). Horses that survive the acute phase may remain seropositive unapparent carriers with low levels of parasitemia (Wise et al., 2013). The chronic syndromes are clinically poorly defined, but are associated with anemia and weight loss. Animals that develop the chronic form of the disease and are asymptomatic constitute a source of infection for ticks (Schnittger et al., 2012; Wise et al., 2013; Scoles and Ueti, 2015). Diagnosis of equine piroplasmosis is challenging because the symptoms are often non-specific, causing significant economic loss in the world´s equine production system (Schnittger et al., 2012).

In Brazil, *Dermacentor nitens* transmits *B. caballi* transovarially (Kerber et al., 2009). In contrast, *T. equi* is maintained by transstadial survival by *Amblyomma sculptum*, which belongs to the *A. cajennense* complex (Kerber et al., 2009; Nava et al., 2014). Molecular evidence suggests that *Rhipicephalus* (*Boophilus*) *microplus* can maintain *T. equi* and *B. caballi* infection by both transovarial transmission and transstadial survival (Battsetseg et al., 2002).

The Pantanal biome is a large floodplain in South America that covers 140,000 km^2. In this area, extensive livestock production is the main economic activity. The Pantanal is estimated to contain four million cattle and four thousand horses (Seidl et al., 1998), which are fundamentally important for cattle management and share the same habitats as the abundant wildlife. Ixodid ticks have been found in the Pantanal in domestic and free-living vertebrates (Ramos et al., 2014, 2016), and equine piroplasmosis has been reported in the north of the region (Barros et al., 2015). Since these parasites could have major economic consequences, we aimed to assess equine piroplasmids infection in the southern Pantanal, the indicators of anemia of infected horses, and their ticks infestation.

2. Materials and methods

2.1. Collection site and field procedures

This work was performed in the central region of the southern Pantanal, the Nhecolândia sub-region (18°59′15″S, 56°37′03″W). This region is characterized by distinct wet and dry seasons (Junk et al., 2006). During October 2015, blood, serum samples and ectoparasitic ticks were collected from 170 horses that had been born and raised in the region. The horses were sampled by a non-probabilistic method (Stevenson, 2005) and divided into four categories according to age, sex, and reproductive status. There were 95 working horses (animals used to management with cattle subject to physical effort due to the long distances they roam, often in flooded areas) above five years of age (41 males and 54 mares), 39 breeding mares, and 36 foals. All of the animals sampled appeared asymptomatic for equine piroplasmids at the time of sampling. All of the procedures were approved by the Ethics Committee for the Use of Animals of the Universidade Católica Dom Bosco (UCDB), according to Protocol No. 021/2015.

During the first six hours after blood sampling with ethylenediaminetetraacetic acid (EDTA), the packed cell volume (PCV) was quantified by the microhematocrit technique and a red blood cell (RBC) count was performed in a Neubauer chamber (Voigt, 2000). The PCV and the RBC count were used as indicators of anemia. Peripheral blood was collected and smears were stained by the panoptic staining method (Hematocor®, Biolog®, Biológica Comercial Ltda., São Paulo, SP, Brazil), according to the manufacturer's recommendations.

Ticks parasitizing horses were collected after a visual inspection for 1 min and identified using previously published dichotomous keys (Barros-Battesti et al., 2006; Nava et al., 2014). The hemolymph and eggs of engorged tick females, after staining with panoptic, were examined under a light microscope with a total amplification of 1000X (Burgdorfer, 1970; Ângelo et al., 2010).

2.2. Enzyme-linked immunosorbent assay (ELISA) for equine piroplasmids

In order to detect IgG antibodies against to *T. equi* and *B. caballi*, the serum samples were subjected to ELISA assays using the antigens and anti-horse IgG conjugated from commercial kit (Imunodot Diagnostics, IMUNOTEST®), following the manufacturer's instructions. Serum samples from positive and negative donkeys were used as controls in the ELISA assays (Machado et al., 2012). We used an ELISA reader (Dynex Technologies) with the cut-off values (0.392 for *T. equi* and 0.538 *B. caballi*) determined as 2.5 times the mean absorbance values of negative controls, and readings above the cut-off value were considered positive (Machado et al., 1997).

2.3. Molecular procedures

DNA extraction from whole blood was performed using the QIAamp DNA Blood Mini Kit (Qiagen), according to the manufacturer's recommendations. The DNA concentration and absorbance ratio (260/280 nm) were measured using a spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA).

In order to perform the molecular detection of equine piroplasmid, the DNA samples were submitted to previously described protocols targeting 1600 bp of the 18 rRNA gene by Nijhof et al. (2005), using "catch-all primers" *Theileria/Babesia*genus-specific (Nbabesia-1 F and 18SRev-TB) (Table 1) (Bhoora et al., 2009). *Theileria equi* and *B. caballi* DNAs obtained from naturally infected animals were used as positive controls in the PCR assays (Machado et al., 2012). Ultrapure sterilized water was used as a negative control.

For the phylogenetic analysis, three nested PCRs (nPCRs) targeted to 18 rRNA gene were performed using the primers listed in Table 1 (Bhoora et al., 2009). Among the 67 amplicons obtained in nested PCR assays, we selected for sequencing thirty positive samples in both molecular and serological tests of different groups. These samples were purified using a Silica Bead DNA Gel Extraction Kit (Thermo Fisher

Table 1
Oligonucleotide sequences used in molecular assays for piroplasmids in equines sampled in the Pantanal wetland, based on the 18S rRNA gene.

Oligonucleotides	Sequences (5'-3')	Base Pairs	Reference
NBabesia1F	AAGCCATGCATGTCTAAGTATAAGCTTTT	29	Oosthuizen et al. (2008)
18SRev-TB	GAATAATTCACCGGATCACTCG	22	Matjila et al. (2008)
BT18S3F	GGGCATTCGTATTTAACTGTCAGAGG	26	Oosthuizen et al. (2008)
BT18S3R	CCTCTGACAGTTAAATACGAATGCCC	26	Unpublished
BT18S2F	GGGTTCGATTCCGGAGAGGG	20	Oosthuizen et al. (2008)
BT18S2R	CCCGTGTTGAGTCAAATTAAGCCG	24	Matjila et al. (2008)

Table 2

Seropositivity and molecular detection for *Theileria equi* and *Babesia caballi* and infestation by ticks in equines in the southern Pantanal wetland. Results are expressed as the total number of animals sampled followed by the percentage of positivity in parentheses.

Categories	N	*T. equi* seropositivity	CI (95%)	*B. caballi* seropositivity	CI (95%)	Co-infection seropositivity	CI (95%)	Molecular occurence	CI (95%)	Presence of ticks
Services males	41	39 (95.1)^{a}	82–99	34 (82.9)^{a}	67–92	34 (82.9)	67–92	6 (14.6)^{a}	6–30	7 (17.1%)
Working females	54	44 (81.5)^{a}	68–90	44 (81.5)^{a}	68–90	42 (77.7)	64–88	14 (25.9)^{a}	15–40	24 (44.4%)
Breeding females	39	16 (41.0)^{b}	26–58	8 (20.5)^{b}	10–37	6 (15.3)	6–31	23 (59.0)^{b}	42–74	34 (87.2%)
Foals	36	6 (16.7)^{c}	7–33	4 (11.1)^{c}	4–27	2 (5.5)	1–20	31 (86.1)^{c}	70–95	23 (63.9%)
Total	170	105 (61.8)	54–69	90 (52.9)	45–61	84 (49.4)	42–57	74 (43.5)	36–51	88 (51.7)

Different letters correspond to statistically significant values at $p \leq 0.05$; N = number of samples tested; 95% CI = confidence interval.

Scientific), in accordance to the manufacturer's recommendations. The amplicons were sequenced in both directions using an automated system based on the dideoxynucleotide chain-termination method (Sanger et al., 1977) at the Center for Biological Resources and Genomic Biology (CREBIO – FCAV – UNESP, Jaboticabal, SP, Brazil).

The quality of the electropherograms obtained through the sequencing of the amplified products was visually analyzed using the program FinchTV 1.4.0. In addition, consensus sequences were obtained by analysis of the forward sequences using the CAP3 program (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py). In order to perform sequence identity analysis, the amplified gene fragments were compared with those previously deposited in GenBank using the basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Finally, our sequences were individually aligned with other sequences available from GenBank using ClustalW (Thompson et al., 1994) and adjusted with BioEdit v.7.0.5.3 (Hall, 1999). Phylogenetic analyses with maximum likelihood (ML) criteria were conducted using RAxML-HPC BlackBox (7.6.3) (Stamatakis et al., 2008) through the CIPRES Science Gateway (Miller et al., 2010). The AICs (Akaike Information Criterion) values obtained by sequence analysis in the MEGA 5.05 program were used to select the best-fit model of evolution. The GTR + G + 1 model was selected and used in the analysis.

2.4. Statistical analysis

A Chi-squared test was used to identify significant differences in seropositivity and molecular characteristics among the horse categories (working males, working mares, breeding mares, and foals). Furthermore, we used the Yates correlation to determine which categories were different from each other.

We used the Shapiro-Wilk test to check the normality of the PCV and RBC results, and analyses of variance (ANOVAs) were conducted to test for significant differences in the PCV and RBC values among the different categories. ANOVAs were also used to test for significant differences in the number of seropositive and seronegative animals for *B. caballi* and *T. equi* among the categories. The Tukey test was then performed in order to ascertain in which groups there were significant differences.

Subsequently, we created a set of models to determine the covariates that would be influencing active infections, expressed by positive results for PCR. The following combinations were used to create the models: (i) Categories: working males, working mares, breeding mares and foals; (ii) Serology: results from ELISA; (iii) Tick: exposure of horses for ticks; (iv) Anemia: PCV and RBC values; (v) Full model: all combinations; and (vi) Null model. Since PCV and RBC values were highly correlated, we combined their results using principal component analysis (PCA). The models were analyzed by Generalized Linear Models (GLM). The candidates for the model and influence of the explanatory variables in the models were compared according to Akaike (1974) and Cruz et al. (2015). The One-Sample *t*-test was used to compare the mean PCV and RBC values of the positive horses (serologically coi-infected and positive according to the PCR) in each category of animal sampled, with the PCV and RBC values presented by Ribeiro et al. (2008) for horses of the Pantaneiro breed.

To determine whether there were significant correlations between the serological and molecular variables, the kappa statistic was calculated. The analyses were performed in the following combinations: (A) PCR vs serology for *T. equi*; (B) PCR vs serology for *B. caballi*; (C) serology for *T. equi* vs serology for *B. caballi*; and (D) PCR vs serology for both *T. equi* and *B. caballi*. All analyzes were performed with the R Development Core Team (2015) and we considered a significance of 95% ($p < 0.05$).

3. Results

Of the 170 animals sampled, 105 (61.8%) and 90 (52.9%) were seropositive for *T. equi* and *B. caballi*, respectively (Table 2). A total of 84 (49.4%) animals were seropositive for both piroplasmids (Table 2). We found 59 animals seronegative for both piroplasmids, while 21 equines were seropositive for only *T. equi* and six were seropositive for only *B. caballi*. Working males and mares had a higher seroprevalence for *T. equi* (95.1% and 81.5%, respectively) than breeding mares (41%) or foals (16.7%) (Table 2). Similarly, working males and mares had a higher percentage of seropositive animals for *B. caballi* (82.9% and 81.5%, respectively) than breeding mares (20.5%) or foals (11.1%) (Table 2).

Seventy-four of the 170 horses sampled (43.5%) were positive by PCR for piroplasmids based on the 18S rRNA gene. Breeding mares and foals had higher percentages of PCR positive animals (59% and 86.1%, respectively) when compared to working males (14.6%) and mares (25.9%) (Table 2).

The combined results of the molecular and serological assays indicated that 21 (12.3%) animals were co-infected with *B. caballi* and *T. equi*. The statistical analyses were not performed on co-infected animals due to the low number of animals in each category.

Concordance was found in the serological tests between *T. equi* and *B. caballi* (kappa value = 0.678). However, no concordance was found between the molecular and serological tests (kappa value < 0). We found different patterns of infection between groups: Of the 59 animals that were seronegative for both *B. caballi* and *T. equi*, 41 (69.5%) were PCR positive; 88% of this group was composed of breeding mares and foals. Of the animals that were seropositive for both *B. caballi* and *T. equi* (n = 84), 63 (75%) tested negative by PCR; 98% of this group was working males and mares.

Regarding the hematological indices, the ANOVA revealed significant differences among the four categories for PCV (df = 03, F value = 6.738, $p = 0.000257$) and RBC (df = 03, F value = 7.439, $p = 0.000105$). When analyzed by the Tukey test, the mean PCV and RBC values for foals were significantly higher than those for the other categories ($p < 0.05$). Using ANOVA, no significant difference in the mean PCV or RBC values between positive and negative animals in each category was found. Similarly, no significant difference was found in the mean PCV or RBC values between co-infected and negative animals sampled in the present study, or for the mean PCV and RBC values of

Table 3
Models used to explore the covariates that influenced active infections, expressed by positive results for PCR, in horses at Brazilian Pantanal. K = Number of Parameters; AICc = Akaike Information Criterion; ΔAICc = Delta Akaike Information Criterion; AICcWt = Akaike Weight; Cum.Wt = Cumulative Akaike Weight.

Model	K	AICc	ΔAICc	AICcWt	Cum.Wt
Categories[a]	3	5	193.52	0	0.95
Serology[b]	4	5	222.67	29.14	0
Tick[c]	5	3	239.92	46.4	0
Anemia[d]	6	3	250.03	56.51	0
Full[e]	2	9	199.23	5.7	0.05
Null[f]	1	2	247.97	54.45	0

[a] Categories: working males, working mares, breeding mares and foals.

[b] Serology: result of ELISA for equine piroplasmids.

[c] Tick: exposure of horses to ticks.

[d] Anemia: results combined by principal component analysis for PCV and RBC.

[e] Full: all combinations.

[f] Null model.

each category as reported by Ribeiro et al. (2008) for horses of the Pantaneiro breed.

The analysis of the multivariable models show that the most parsimonious model was Categories (Table 3), breeding mares and foals presented positive influence on PCR positivity since confidence intervals did not overlap zero.

We found that 51.8% (88/170) of the horses evaluated were infested with ticks. Breeding mares had the highest infestation (87.2% [34/39]), followed by foals (63.9% [23/36]), working mares (44.4% [24/54]) and males (17.1% [7/41]) (Table 4).

Dermacentor nitens was found in all of the categories evaluated. Breeding mares and foals had the highest infestation by *D. nitens* (35.9% and 27.9%, respectively). Breeding mares also had the most occurrences of co-infestation by two (*A. sculptum* and *D. nitens*) or three (*R.* [*B.*] *microplus*, *A. sculptum*, and *D. nitens*) ixodid tick species (Table 4). We did not find sporokinets in the hemolymph of engorged ticks female, and no structures suggestive of piroplasmids were found in the equines blood smears.

From 180 sequences (forward and reverse nucleotide sequences comprising 3′, inner and 5′ fragments of 18S rRNA gene) obtained, 54 were recovered, 18 contigs were formed and properly deposited at GenbBank with (access numbers KY464019 to KY464036). BLAST analyses of the contigs revealed an identity ranging from 98 to 100% with previously amplified *T. equi* sequences in the USA (JX177673) and Brazil (KU240071).

Theileria equi sequences clustered in two main groups (Fig. 1). The sequences belonging to group A were subdivided into two subgroups (A1 and A2). Four sequences from subgroup A1 (JC9, JC16, JC28 and JC29) grouped with sequences detected in equids from Rio de Janeiro, southeastern Brazil (KJ573370), Rio Grande do Sul, southern Brazil (KU240065, KU24008, KU240064, KU240072 and KU240069), South Africa (EU888906), and the United States of America (JX177673, JX177671, JX177672 and JX177670). Five sequences of subgroup A2 (JC2, JC3, JC6, JC7 and JC12) were closely positioned to *T. equi* detected in Rio de Janeiro, southeastern Brazil (KJ573373), South Africa (EU642508) and Spain (AY150062 and AY150063). In addition, group B genotypes were subdivided into two subgroups (B1 and B2). Seven sequences (JC4, JC10, JC15, JC17, JC17, JC19 and JC20) belonging to subgroup B1 were closely positioned to those detected in Rio de Janeiro (KJ573371), Rio Grande do Sul (KU240067 and KU240071) and United States (JQ390047). Two sequences of subgroup B2 (JC18 and JC21) clustered closely to those sequences detected in Rio Grande do Sul (KU240066 and KU240070), Rio de Janeiro (KJ573374), South Africa (EU888905, EU642511, EU888903, EU642510), and Sudan (AB515309, AB515312, AB515307, AB515315) (Fig. 1).

4. Discussion

This is the first work that broadly evaluates the epidemiology of equine piroplasmosis in the main Brazilian cattle ranch industry, Pantanal region. The endemic character of equine piroplasmids in the Pantanal wetland was demonstrated by seropositivity rates of 61.8% and 52.9% for *T. equi* and *B. caballi* respectively, positivity of 43.5% in PCR, and 51.2% of ticks infestation in the horses sampled.

Serological tests are essential in epidemiological studies regarding equine piroplasmosis because they detect animals with cryptic parasitemias. Sero-epidemiological studies performed in southeastern and midwestern Brazil have reported higher seropositivity rates for *T. equi*, such as in the states of Rio de Janeiro (90.6% by indirect fluorescent antibody test [IFAT]), Minas Gerais (91% by IFAT), São Paulo (73.8% by ELISA), and Paraná (78.3% by cELISA) (Pfeifer et al., 1995; Heim et al., 2007; Machado et al., 2012; Vieira et al., 2013). Similarly, a higher rate of seropositivity for *B. caballi* was reported among horses sampled in the states of São Paulo and Mato Grosso do Sul (90% by latex agglutination test and ELISA) (Xuan et al., 2001). These regional differences in seropositivity should be evaluated with caution due to environmental local characteristics, horse management particularities, as well as different serological tests standardization (Barros et al., 2015; Davitkov et al., 2016; Liu et al., 2016).

The slightly higher seropositivity for *T. equi* than *B. caballi* found in the present study has been previously reported in Brazil (Heim et al., 2007; Vieira et al., 2013; Ferreira et al., 2016) and worldwide (Camacho et al., 2005; Karatepe et al., 2009; Garcia-Bocanegra et al., 2013). This finding can be attributed to long-lasting *T. equi* infections, whereas *B. caballi* infections usually disappear after four to five years (Vial and Gorenflot, 2006; Rüegg et al., 2007 and 2008). Moreover, treatment seems to be particularly more effective against *B. caballi* than *T. equi* (Brüning, 1996; Schwint et al., 2009).

Our results showed that working males and mares displayed significant higher seroprevalence rates than breeding mares or foals. The lower seroprevalence found in breeding mares, may be associated with immunossupression caused by the stress of feeding and caring for their foals (Cohen et al., 2012). Indeed, previous research has shown that a

Table 4
Single and co-infestations by ticks in horses sampled in the southern Pantanal wetland. Data are presented as the number of infested horses in different categories, followed by the percentage of occurrence.

Tick Species	Working Males (N = 41)	Working Mares (N = 54)	Breeding Mares (N = 39)	Foals (N = 36)
Dermacentor nitens	5 (12.2)	13 (24.1)	14 (35.9)	12 (27.9)
Amblyomma sculptum	2 (4.9)	3 (5.6)	8 (20.5)	–
Rhipicephalus (Boophilus) microplus	–	1 (1.9)	–	5 (13.9)
R. (B.) microplus + D. nitens	–	2 (3.7)	1 (2.6)	4 (11.1)
A. sculptum + D. nitens	–	3 (5.6)	4 (10.3)	1 (2.8)
R. (B.) microplus + A. sculptum	–	1 (1.9)	1 (2.6)	1 (2.8)
R. (B.) microplus + A. sculptum + D. nitens	–	1 (1.9)	6 (15.4)	–
Total	7 (17.1)	24 (44.4)	34 (87.2)	23 (63.9)

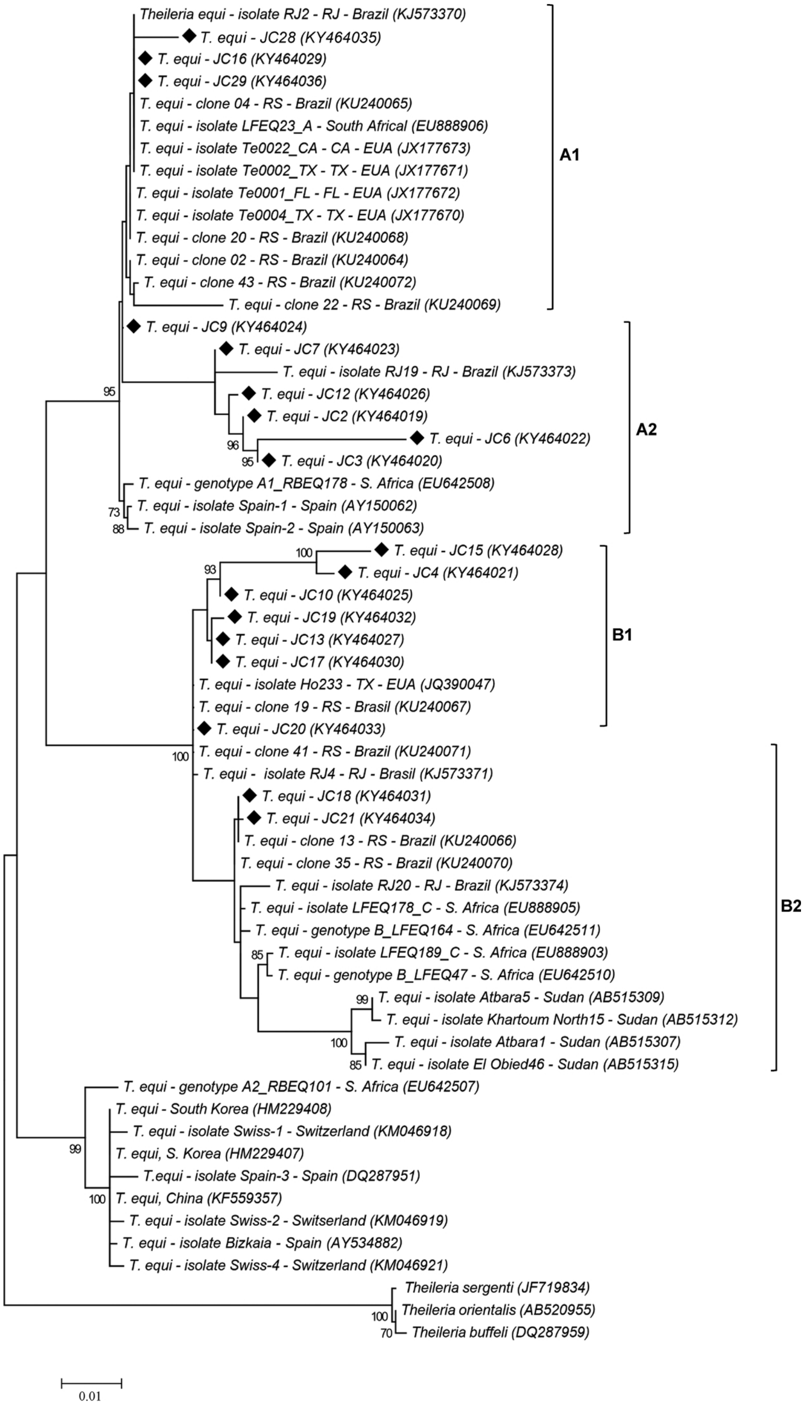

Fig. 1. Phylogenetic tree of *Theileria equi* 18S rRNA gene sequences. Analysis was performed using the maximum likelihood method implemented with the GTR + G + I substitution model. Numbers in the branches correspond to bootstrap values after 1000 repetitions. 18S rRNA sequences of *T. sergenti*, *T. orientalis*, and *T. buffeli* were used as outgroups (◆) sequences obtained in this study.

persistent stressful situation, such as care for offspring, is associated with chronic increases in cortisol and corticosterone levels in the plasma, resulting in a decrease in lymphoid cells (Dobson and Smith, 2000; Cohen et al., 2012).

We observed differences in the patterns of infection among the categories of the horses evaluated: (i) the high occurrence of PCR positive foals observed (86.1%) may be related to an acute phase of equine piroplasmosis because foals are exposed to infected ticks since they are born (Battsetseg et al., 2002; Ribeiro et al., 2011; Scoles and Ueti, 2015), and carrier mares may transmit *T. equi* to their apparently healthy offspring (Allsopp et al., 2007; Georges et al., 2011); (ii) adult working males and mares had the lowest PCR prevalence, which was expected, since adults are more efficient in controlling piroplasmid infections than juveniles due to their cell-mediated immunity, resulting in undetectable cryptic parasitemias (Ramsay et al., 2013; Bartolomé del Pino et al., 2016); (iii) over half of the breeding mares (59.0%) tested positive by PCR likely because of chronic reproductive stress, with consequent immunosuppression as above mentioned. The compromised immune systems of breeding mares may also explain why they had the largest tick infestation (87.2%) found in this category. This situation is a vicious cycle, because both reproductive stress and high tick infestations compromise the health and immune system of breeding mares (Cohen et al., 2012).

Our results highlight the importance in using different diagnostic tests to detect piroplasmids in horses in enzootic areas because of the discordance between molecular and serological assays. We found a high percentage of working animals (98.0%) positive by serology for both *B. caballi* and *T. equi*, but negative by PCR. These results could be associated to the fact that the patent parasitemia decreases to low levels as animals age, becoming undetectable by molecular tests, making serology essential for detecting infected animals (Posnett et al., 1991; Holman et al., 1993; Ikadai et al., 2000; Cunha et al., 2006; de Waal and Van Heerden, 2004; Jaffer et al., 2010). Furthermore, 88% of the seronegative animals that displayed positivity in molecular tests included breeding mares and foals, probably related to reproductive stress and acute phase respectively. Disagreement between serological and molecular tests regarding natural infections of equine piroplasmosis has been reported by several authors (Jaffer et al., 2010; Machado et al., 2012; Abutarbush et al., 2012; Vieira et al., 2013). Moreover, as we did not find a correlation between the results of the molecular and serological tests, we strongly suggest the use of both methods for detection of equine piroplasmids for epidemiological studies in enzootic areas since PCR and ELISA assays detect acute and chronic phase of infection respectively.

The presence of *D. nitens*, *A. sculptum* and *R. (B.) microplus* parasitizing horses may be correlated with the occurrence of *T. equi* and *B. caballi* infections (Wise et al., 2013; Scoles and Ueti, 2015). It is noteworthy that the environment where the sampled horses were raised is highly infested with immature forms of tick, particularly the main vector of *T. equi, A. sculptum*, parasite of several wild and domestic mammal species in the studied area (Scoles and Ueti, 2013; Ramos et al., 2014, 2016). Furthermore, it has been reported that equine piroplasmosis is associated with horses that share the same environments with cattle (Garcia-Bocanegra et al., 2013). Therefore, the extensive livestock production system in the Pantanal region may highly favor populations of *R.* (*B.*) *microplus*. Notably, we observed *R.* (*B.*) *microplus* infestations in breeding mares and foals, which would ensure the transmission of *T. equi* (Battsetseg et al., 2002; Ueti et al., 2005).

As expected, and observed here in foals, young mammals commonly have higher anemia indices (PCV and RBC) than those observed for adult individuals (Ribeiro et al., 2008; Parreira et al., 2016). Our findings demonstrated that equine piroplasmids did not affect the mean PCV and RBC values between animals that tested positive and those that tested negative, even when comparing positive animals with standard values for the Pantaneiro breed (Ribeiro et al., 2008). The subclinical profile of equine piroplasmosis was further evident by the lack of parasites found on blood smears. The visual detection of piroplasmids by light microscopy is the most common, simple, and accessible confirmatory diagnostic test, however, the sensitivity of this technique is extremely low and parasites are not detectable in the chronic phase of the disease (Brüning, 1996; Sadeghi Dehkordi et al., 2010). Moreover, the absence of clinical signs in infected horses, associated to 43.5% of positivity in the molecular test suggests that these chronic carriers may act as a source of infection for vectors (Ueti et al., 2005; Liu et al., 2016). In fact, asymptomatic carriers have been reported in previous studies as a major challenge in the control of equine piroplasmids (Camacho et al., 2005; Wise et al., 2013).

The overall positive result in the molecular assays found in this study are in line with previous studies performed in Midwest, Northeast and Southeastern regions of Brazil (Heim et al., 2007; Ferreira et al., 2016). However, in a study conducted in the north Pantanal of Mato Grosso state, only 14% of the 121 horses sampled exhibited positivity in molecular tests (Barros et al., 2015). Extensive sequence variations have been reported in the 18S rRNA gene of piroplasmids (Bhoora et al., 2009; Liu et al., 2016), so caution should be exercised when using this target for diagnostic purposes. However, it is possible to identify a conserved region of equine piroplasmids in the 18S rRNA gene using “catch-all primers” for *Theileria/Babesia*genus-specific (Criado-Fornelio et al., 2003; Davitkov et al., 2016), as we used in the present study.

According to Veronesi et al. (2014), high and constant exposure to piroplasmids favors the acquisition of immunity, turning infected animals into chronic carriers (Garcia-Bocanegra et al., 2013; Veronesi et al., 2014). However, increases in parasitemia may occur in cases of continuous physical effort (Ferreira et al., 2016), which is common for as horses practicing cattle ranching in the Pantanal region. Furthermore, the study area is endemic for equine infection anemia virus (EIAV) and trypanosomiasis caused by *T. evansi* (Seidl et al., 1998; Parreira et al., 2016), the occurrence of coinfections may be a risk factor for the emergence of parasitemias and clinical presentations of equine piroplasmosis.

Through sequencing, this study revealed parasitemias only by *T. equi*, probably due to their higher persistence after infection. Our phylogenetic analyses showed some microheterogeneity among genotypes for *T. equi*, as reported in South Africa (Bhoora et al., 2009), Sudan (Salim et al., 2010), Jordan (Qablan et al., 2013), and North America (Hall et al., 2013). Indeed, the diversity of *T. equi* may increase with the addition of sequences from geographically unexplored regions (Liu et al., 2016). The clinical significance of these genotypes in the presentation of equine piroplasmosis should be further investigated. In addition, the phylogenetic positioning of the different genotypes detected in equids around the world contributes to our understanding of the Piroplasmorida phylogeny.

5. Conclusion

The studied area is enzootic for *B. caballi* and *T. equi* demonstrated by the positivity observed in serological and molecular tests, as well as the high infestation of horses by different species of tick vectors, mainly *A. sculptum*. The methods of equine piroplasmids detection allowed the recognition of different patterns of infection in the sampled horses. In this sense, although working animals showed the highest seroprevalences rates, breeding mares and foals exhibited the highest molecular positivity and ticks infestation and consequently should be closely monitored. Once risk factors for clinical presentation of equine piroplasmosis include continuous physical effort and coinfections, animals used for breeding must undergo special management precautions as periodic tick control, diagnostic for EIAV and trypanosomiasis caused by *T. evansi*, as well strategic deworming.

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