

Serological detection and molecular characterization of piroplasmids in equids in Brazil



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ABSTRACT

Equine piroplasmidosis is a disease caused by the hemoparasites *Babesia caballi* and *Theileria equi* and is considered to be the most important parasitic infection affecting Equidae. The objective of the present study was to carry out an epidemiological molecular and serological survey for the presence of these two protozoal organisms in equids from the northwestern region of the State of Rio Grande do Sul (RS), south Brazil. For this purpose, blood samples were collected from 90 equids in the city of Passo Fundo, RS, Brazil. Those were animals used for sport activities, outdoor recreational riding, and work including cattle herding and mounted patrol. Anti-*T. equi* and anti-*B. caballi* IgG antibodies were detected in the sera of those animals by commercial ELISA kits. The molecular diagnosis of equine piroplasmidosis due to *T. equi* or *B. caballi* (or both) consisted in the amplification of the 18S rRNA gene by nested PCR followed by sequencing of the amplified PCR product and sequence comparison and phylogenetic analysis of the isolates; 17 (18.9%) and 5 (5.55%) out of the 90 serum samples tested in this study were positive for *T. equi* and *B. caballi*, respectively. Piroplasmid 18S rRNA gene fragments were detected by PCR in 24.4% (22/90) of the samples analysed and shared 99–100% identity with sequences of *T. equi* by BLASTn. Samples for the phylogenetic analysis were divided into 2 groups. In group A, there was close phylogenetic relationship between 4 sequences and sequences previously reported along the US-Mexico border, in South Africa, and in Brazil. There was a phylogenetic proximity between 5 samples from group B and samples tested by other authors in the US and Spain. Variation of the 18S rRNA gene allowed the identification of 9 new *T. equi* genotypes in the geographical region studied.

1. Introduction

Equine piroplasmidosis (EP) is caused by the hemoprotozoan parasites *Babesia caballi* (Nuttall and Strickland, 1912) and *Theileria equi* (Laveran, 1901) and is regarded as the most significant parasitic disease affecting the equine industry worldwide (Zobba et al., 2008). The World Organization for Animal Health (OIE) includes equine piroplasmidosis in the list of notifiable diseases, and there are international traffic restrictions for equids infected with *T. equi* and *B. caballi* (OIE, 2017).

EP is transmitted by ticks of the Ixodidae family (Stiller et al., 2002) and iatrogenic transmission and intrauterine infections have also been reported (Getstenberg et al., 1999; Wise et al., 2013). EP is endemic in Asia and many parts of Europe, South and Central Americas and Africa

(OIE, 2014). In the year 2012, 30 countries reported the occurrence of EP (OIE, 2014).

Although *A. cajennense* sensu lato showed to be a competent intra-stadial vector of *T. equi* (Scoles and Ueti, 2013), the transtadial transmission of *T. equi* by this tick species failed to be proved (Ribeiro et al., 2011; Scoles and Ueti, 2013). In Brazil, *T. equi* and *B. caballi* are mainly transmitted by *Rhipicephalus (Boophilus) microplus* and *Anocentor nitens*, respectively (Guimarães et al., 1998; Battsetseg et al., 2002; Kerber et al., 2009).

Brazil is considered endemic for *T. equi* infection (Heim et al., 2007) which prevalence varies according to how horses are raised in the area and which tick species are present in this particular zoogeographic region (Botteon et al., 2002).

The study of the genetic diversity of hemoparasite populations has

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contributed to the understanding of the epidemiology of tick-borne diseases (Allsopp and Allsopp, 2006). Molecular tools have been employed to explore genetic structure and to assess epidemiological monitoring allowing researchers to analyse pathogen populations. For this reason, studies on the phylogenetic classification of *T. equi* have been carried out all over the world such as Spain, South Africa, USA, Korea and Brazil (Bhoora et al., 2009; Ferreira et al., 2016; Hall et al., 2013; Nagore et al., 2004; Seo et al., 2013).

The present study aimed to investigate the prevalence of anti-*T. equi* and anti-*B. caballi* antibodies in the serum of equids raised in the of the northwest region State of Rio Grande do Sul (RS), south Brazil, and to molecularly characterize the piroplasmid isolates that are present in this population.

2. Materials and methods

This study was approved by the Animal Experimentation Ethics Committee and Animal Care and Use Committee from the Federal University of Passo Fundo (UPF) (protocol 024/2014), Passo Fundo, RS, Brazil.

2.1. Samples

Between March and December 2014, blood samples were collected from 90 equids on 4 different premises [1–Inn (Horse Hotel), 2 – City Hall, 3 – Brigade, 4 – Veterinary Hospital of the University of Passo Fundo]. Passo Fundo (28° 15′ 40″ S, 52° 24′ 30″ W) is a county in the northwest region of the State of Rio Grande do Sul (RS), south Brazil (Fig. 1). It is located 680 m above sea level, and includes fields and strips from the Atlantic Forest. The city has a humid subtropical climate, well-distributed rainfall all year round, hot summers, cold

winters, and annual mean temperature of 17.5 °C (Embrapa, 2017). This epidemiological survey was conducted in the following facilities: Inns (horse hotels) from different farms (n = 30) where sampled horses had contact with cattle; Prefecture (n = 14) where animals were raised in the outskirts of the county; Brigade (n = 43) where horses from mounted patrol units were kept in a farm and grazed on native grass pastures; Veterinary Hospital (n = 3) which provided surgical and medical care to animals. At the time of collection of blood samples the animals had no ticks on their coat and were healthy and didn't show any clinical signs consistent with equine piroplasmiasis.

Blood samples were collected from the jugular vein by venipuncture into vacuum blood collection tubes (Vacutainer) with and without EDTA. Serum was obtained by centrifuging blood samples at room temperature (25 °C) at 3000 rpm for 10 min. Serum samples were stored at –20 °C until the serological testing was performed. Collection tubes containing EDTA were stored at –80 °C for further DNA extraction.

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

For the detection of IgG antibodies against *T. equi* and *B. caballi*, serum samples were tested with commercial ELISA kits (Imunodot Diagnostics, IMUNOTEST[®] (specificity and sensitivity:100%; 80% and 84% for *T. equi* and *B. caballi*, respectively) according to the manufacturer's instructions. Briefly, 100 µL of *T. equi* and *B. caballi* antigens (concentrations of 10 µg/mL and 2.5 µg/mL, respectively), were diluted in a 0.05 M sodium bicarbonate-carbonated buffer (pH 9.6) and added to each well of a micro-ELISA plate (Nunclon[™] Surface; Nunc, Denmark). After incubation at 4 °C for 18 h, the excess of antigen was removed by washing the plate with PBS Tween-80 at 0.05%. Uncoated sites in the wells were blocked by adding 200 µL of PBS Tween-80 containing 6% skim milk for 2 h at 37 °C. The blocking agent was

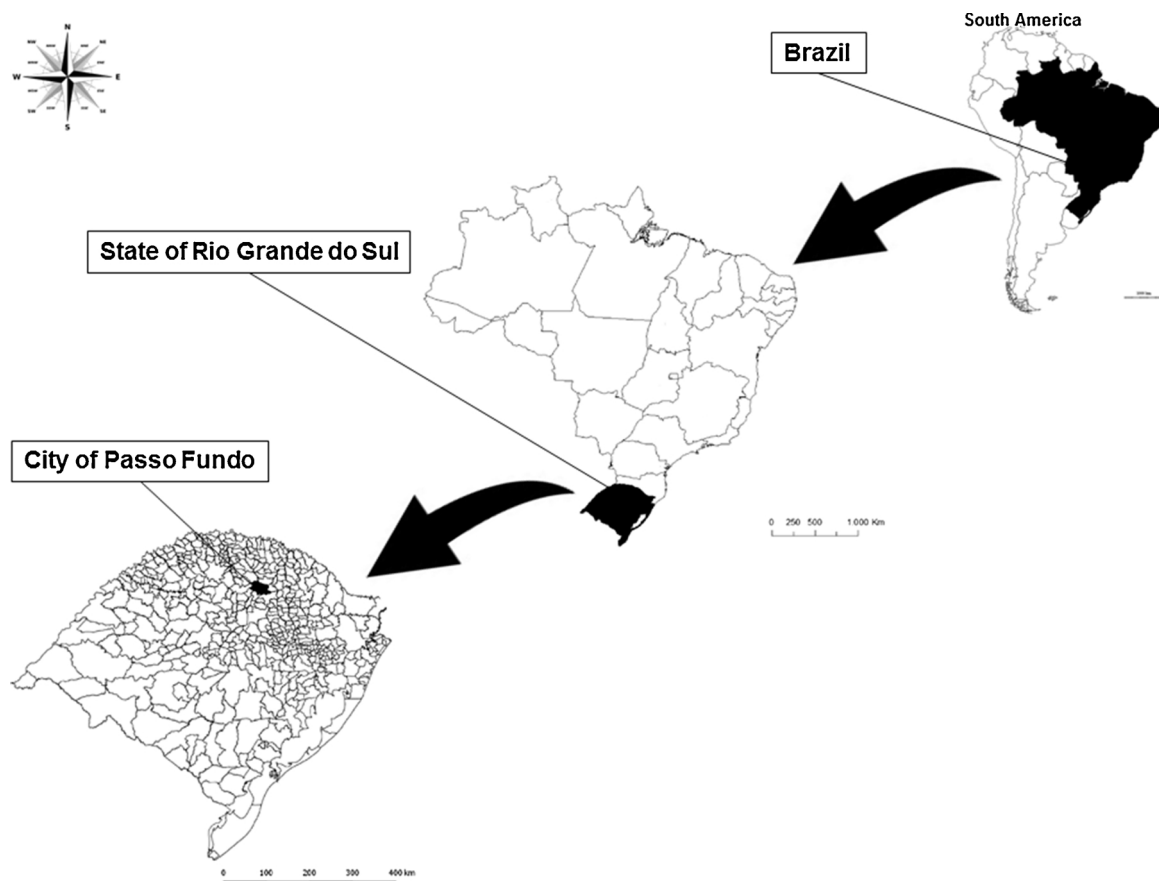


Fig. 1. Geographic location in the northwestern region of the State of Rio Grande do Sul (RS), southern Brazil, where equids were sampled in the present study.

removed, the plate was washed with PBS Tween-80 at 0.05%, and individual equid sera diluted (1:100) in PBS Tween-80 with 5% skim milk (PBS-TWSM) were added to each well and then incubated for 90 min at 37 °C. After washing the plates, 100 µL of alkaline phosphatase anti-horse IgG conjugated (Sigma–Aldrich, St. Louis, Missouri, USA) diluted 1:30.000 in PBS-TW-SM were added to each well and then incubated for 90 min at 37 °C. The plates were washed and the appropriate substrate (*p*-Nitrophenyl phosphate) (Sigma–Aldrich, St. Louis, Missouri, USA) was added. Serum samples of donkeys from previous studies that tested positive or negative for equine piroplasmosis by ELISA were used in this study as known positive and negative controls in the ELISA assays (Machado et al., 2012). Absorbance at 405 nm was read after 45-min incubation at room temperature using an ELISA reader (Dyner Technologies). The cut-off value (0.318 for both *T. equi* and *B. caballi*) was determined as being 2.5 times the mean absorbance value of negative controls, and readings above the cut-off value were considered positive according to (Machado et al., 1997).

2.3. DNA extraction

DNA was extracted from blood samples with EDTA using a QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

2.4. Molecular detection and characterization of *T. equi* e *B. caballi*

Previously published conventional PCR protocols based on the 18S rRNA gene (1600 bp) were used to amplify *T. equi* and *B. caballi* DNA (Bhoora et al., 2009) (Fig. 2). The primers (namely, NBabesia1F: 5'-AAGCCATGCATGTCTAAGTATAAGCTTTT-3' and 18SRev-TB: 5'-GAATAATTCACCGGATCACTCG-3') were used for this purpose (Table 1). The reactions were performed using 5 microliters of DNA as a template in 25 µL reaction mixtures containing 10X PCR buffer, 1.0 mM MgCl₂, 0.8 mM deoxynucleotide triphosphate (dNTPs) mixture, 1.0 U Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA) and 0.5 µM of each primer (Integrated DNA Technologies). PCR amplifications were performed by an initial cycle of 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 45 s and 72 °C for 1 min, and a final extension of 72 °C for 7 min. In addition, DNA samples were submitted to another 3 nPCR assays in order to obtain the whole 18S rRNA gene fragment of *T. equi* and *B. caballi*. The amplification conditions were the same as previously described except for the annealing temperature (Table 1). DNA of *T. equi* and *B. caballi* from naturally infected donkeys were used as positive controls (Machado et al., 2012). Ultra-pure sterile water was used as the negative control in all PCR assays as detailed above. In order to avoid PCR contamination, DNA extraction, reaction

setup, PCR amplification and electrophoresis were performed in separated rooms. The gels were imaged under ultraviolet light using the Image Lab Software version 4.1 (Bio-Rad®). The amplified fragments of the expected size for each nested PCR assay were purified from the gel using Silica Bead DNA Gel Extraction Kit (Fermentas, São Paulo, SP, Brazil).

2.5. Sequencing and phylogenetic analysis

Amplicons obtained from nested PCR assays were sequenced in both directions using the same PCR primers (forward and reverse) by Sanger sequencing at (ABI Prism 310 Genetic Analyser–Applied Biosystem/Perkin Elmer). Consensus sequences were obtained through analysis of the sense and antisense sequences using the CAP3 program (<http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py>). Sequences deposited in the GenBank database were compared using the basic local alignment search tool (BLASTn) (Altschul et al., 1990). The sequences were aligned with sequences published in GenBank using Clustal/W (Thompson et al., 1994) and manually adjusted in Bioeditv.7.0.5.3 (Hall, 1999). Phylogenetic inference based on Maximum Likelihood criterion (ML) was inferred with RAXML-HPC BlackBox 7.6.3 (Stamatakis et al., 2005) through the CIPRES Science Gateway (Miller et al., 2011). Akaike information criterion (AIC) was used in Mega 5.05 to identify the most appropriate model of nucleotide substitution. GTRGAMMA + I model was chosen as the most appropriate for the Maximum Likelihood analysis of the 18S rRNA sequence alignment. The sequences amplified in the present study were deposited in the GenBank database under accession numbers: KU240064 to KU240072.

2.6. Statistical analysis

Descriptive statistics was used in the analysis of all data generated by serology and nPCR, and was expressed in absolute (total) and relative (percentage) frequencies. The software package SPSS® Statistics version 20.0 was used to analyse the data.

3. Results

None of the equids included in this study presented clinical signs of piroplasmosis or were infested with ticks at the time of blood sampling. Seventeen (18.9%) and 5 (5.55%) horses were seropositive for *T. equi* and *B. caballi*, respectively (Table 2). Ten (11.11%) equids were positive for *T. equi* by both ELISA and nPCR assays. Seven (7.8%) animals were seropositive for *T. equi* by ELISA but negative in nPCR assays for this piroplasmid species (Table 3). On the other hand, 12 (13.33%) animals were seronegative for *T. equi* by ELISA but positive for *T. equi*

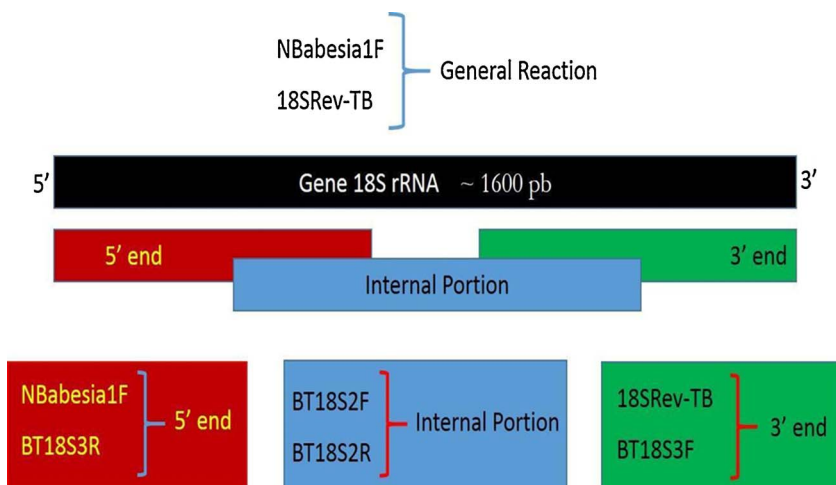


Fig. 2. Nested PCR protocols based on the 18S rRNA gene (1600 bp) used to amplify *Theileria equi* and *Babesia caballi* DNA, according to Bhoora et al. (2009).

Table 1
Sequences of the primers used to amplify 18S rRNA gene of equine piroplasmids.

Primers	Reactions in PCR protocol	Oligonucleotide sequences (5'-3')	Annealing temperature	References
NBabesia1F	5' end	AAGCCATGCATGTCTAAGTATAAGCTTTT	58 °C	Oosthuizen et al. (2008)
BT18S3R	5' end	GAATAATTCACCGGATCACTCG		Matjila et al. (2008)
BT18S2F	Internal Portion	GGGTTCGATTCCGGAGAGGG	55 °C	Oosthuizen et al. (2008)
BT18S2R	Internal Portion	CCCGTGTGAGTCAAATTAAGCCG		Matjila et al. (2008)
BT18S3F	3' end	GGGCATTCGTATTTAACTGTCAGAGG	55 °C	Oosthuizen et al. (2008)
18SRev-TB	3' end	CCTCTGACAGTTAAATACGAATGCC		Bhoora et al. (2009)

Table 2
Serological and molecular prevalence of *T. equi* and *B. caballi* in equids from southern Brazil.

	ELISA for <i>Theileria equi</i>	ELISA for <i>Babesia caballi</i>	nPCR for <i>Theileria equi</i>
Positive	17 (18.9%)	5 (5.6%)	22 (24.4%)
Negative	73 (81.1%)	85 (94.4%)	68 (75.6%)
Total	90 (100%)	90 (100%)	90 (100%)

Table 3
Comparison between the results of ELISA and nPCR assays for *T. equi* in equids from southern Brazil.

	ELISA <i>Theileria equi</i>		Total
	Positive	Negative	
nPCR <i>Theileria equi</i>			
Positive	10 (11.1%)	12 (13.3%)	22 (24.4%)
Negative	7 (7.8%)	61 (67.8%)	68 (75.6%)
Total	17 (18.9%)	73 (81.1%)	90 (100.0%)

Table 4
Comparison between the results found in ELISA tests for *B. caballi* and *T. equi* in equids southern Brazil.

	ELISA <i>Babesia caballi</i>		Total
	Positive	Negative	
ELISA <i>Theileria equi</i>			
Positive	2 (2.2%)	15 (16.7%)	17 (18.9%)
Negative	3 (3.3%)	70 (77.8%)	73 (81.1%)
Total	5 (5.6%)	85 (94.4%)	90 (100.0%)

Table 5
Comparison between the results of ELISA for *B. caballi* and nPCR assays for *T. equi* in equids from southern Brazil.

	ELISA <i>Babesia caballi</i>		Total
	Positive	Negative	
nPCR <i>Theileria equi</i>			
Positive	1 (1.1%)	21 (23.3%)	22 (24.4%)
Negative	4 (4.4%)	64 (71.1%)	68 (75.6%)
Total	5 (5.6%)	85 (94.4%)	90 (100.0%)

by nPCR. Additionally, 2 (2.22%) animals were seropositive for both *T. equi* and *B. caballi* (Table 4). One (1.11%) animal was positive for both *T. equi* by nPCR and *B. caballi* by ELISA but seronegative for *T. equi* (Table 5).

Among all equid DNA samples that were analysed, 24.4% (22/90) were positive for equine piroplasmids in 18S rRNA-based nPCR assays. All 22 sequences shared identities ranging between 99% and 100% with *T. equi* isolates previously detected by other researchers by BLASTn analysis in the USA and on the US-Mexico border (Table 6). *B. caballi* DNA was not detected in any of the samples sequenced. Additionally, 9 sequences were submitted to phylogenetic analysis based on a 1600pb 18S rRNA gene fragment, since that the amplification of all three 18S rRNA fragments (5', inner and 3' regions) was not achieved in 13 samples

The sequences were included into 3 major groups (#1, #2 and #3), which were then subdivided into 9 clusters (A-H) (Fig. 3). Among the 9 sequences amplified, 2 clustered with other 2 *T. equi* sequences previously detected in equids in the State of Rio de Janeiro (RJ), southeast Brazil, and with 1 sequence reported by other authors in South Africa, forming Group 1, cluster C; 2 sequences were positioned near the *T. equi* sequence previously detected in a horse on the USA-Mexico border, establishing Group 1; cluster E; 5 sequences, belonging to Group 3 and cluster I, were closely related to *T. equi* isolates previously detected in the USA, Spain, South Africa, and Brazil (Fig. 3).

4. Discussion

The use of serological tests such as ELISA has been recommended for the diagnosis of equine piroplasmids. Serologic surveillance would prevent seropositive animals from entering piroplasmids-free countries such as the US, Canada, Australia, Japan, and New Zealand (Brüning, 1996). In the present study, 18.9% and 5.55% of equids were seroreactive for *T. equi* and *B. caballi*, respectively. Previous serosurveys of horses for antibodies to *T. equi* and *B. caballi* in south Brazil have shown prevalence rates for *T. equi* ranging between 31.6% and 46.25% (Dall'Agnol et al., 2013; Golynski et al., 2008).

The results of previous seroprevalence surveys for equine piroplasmids carried out in Brazil show that in those regions of the country that are considered endemic for the disease such as the State of Minas Gerais (MG), southeast Brazil, the frequency of serum antibodies against *T. equi* and *B. caballi* ranges between 83% and 91%, respectively (Heim et al., 2007). On the other hand, in the city of Jaboticabal, State of São Paulo (SP), southeast Brazil, 100% of the horses tested were seropositive for *T. equi* (Baldani et al., 2010). In Orlandia county, state of São Paulo (SP), southeast Brazil, 73.8% and 93.2% of the serum samples from donkeys were positive for *T. equi* and *B. caballi*, respectively. In the State of Paraná (PR), south Brazil, 61% of the animals were seropositive for *T. equi* in a study population of 400 horses (Prochno et al., 2014). In the State of Rio Grande do Sul (RS), Brazil, blood samples from 108 mares were collected in a premise located in the south of the state; 22% and 15% of these samples were positive for *T. equi* by Indirect Immunofluorescent Test (IFAT) and by nPCR, respectively (Nizoli et al., 2008).

Although equids from the study population were asymptomatic, animals from the Horse Hotel, the Brigade, and the City Hall were seropositive for *T. equi* or *B. caballi* (or both) or the 18S rRNA gene was PCR-amplified. These results are epidemiologically relevant as they reinforce the fact that asymptomatic, infected carriers are possibly the source of infection for tick vectors, perpetuating piroplasmids in equine populations.

In the present epidemiological survey, 7 horses were positive for *T. equi* by ELISA and negative for *T. equi* by nPCR. Those are possibly carrier animals chronically infected with the piroplasm and in which the parasitemia is below the detection threshold of molecular diagnostic techniques. A low-grade, long-lasting parasitemia would stimulate the immune system in animals maintaining antibodies in serum at detectable levels. On the other hand, induction of a protective antibody response in animals previously exposed to *T. equi* and that cleared the infection is a possibility that should also be considered. *T. equi* DNA was

Table 6

Nine isolates of equine piroplasms whose 18S rRNA sequences were used in phylogenetic analysis according to their location, host and GenBank accession number.

Samples	Accession Number	Animals	% Identity	Genotype	References
Isolate02	KU240064	Female horse	99	JX177670 Texas – USA	Hall et al. (2013)
Isolate04	KU240065	Female horse	99	JX177673 Texas – USA	Hall et al. (2013)
Isolate13	KU240066	Male horse	99	JQ390047 USA-Mexico	Hall et al. (2013)
Isolate19	KU240067	Female horse	100	JQ390047 USA-Mexico	Hall et al. (2013)
Isolate20	KU240068	Male horse	100	JX177670 Texas – USA	Hall et al. (2013)
Isolate22	KU240069	Female horse	99	CP001669 – USA	Kappmeyer et al. (2012)
Isolate35	KU240070	Male horse	99	JQ390047 USA-Mexico	Hall et al. (2013)
Isolate41	KU240071	Male Horse	100	JQ390047 USA-Mexico	Hall et al. (2013)
Isolate43	KU240072	Female horse	99	CP001669 – USA	Kappmeyer et al. (2012)

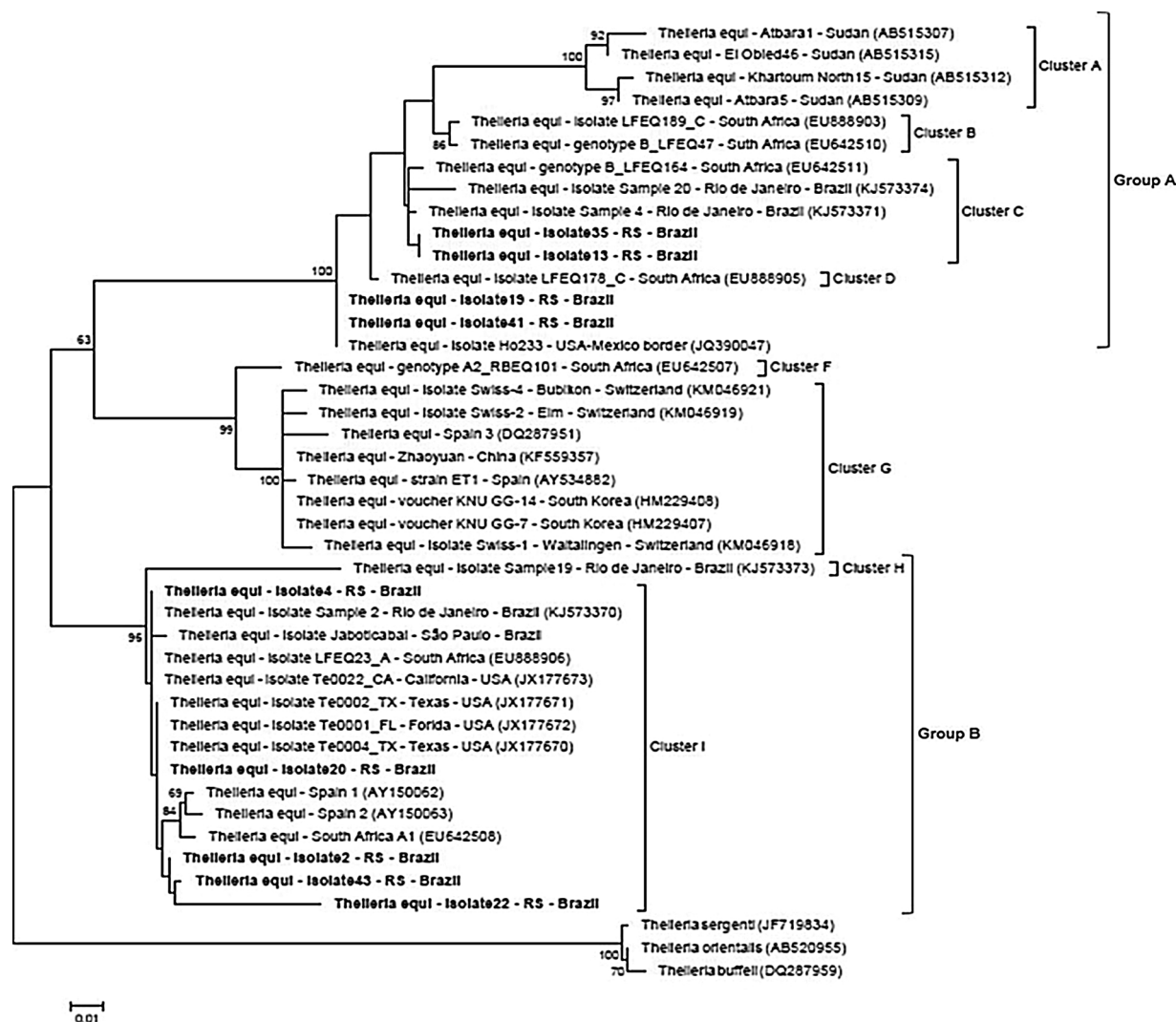


Fig. 3. Phylogenetic relationships within the *Theileria equi* sequences based on a 1600 bp fragment of the 18S rRNA gene. The phylogenetic tree was inferred by using the maximum likelihood method. The sequences amplified in the present study are highlighted in bold. The numbers at the nodes correspond to bootstrap values higher than 60% accessed with 1.000 replicates. *Theileria* species was used as outgroup. RS: Rio Grande do Sul.

detected by PCR in blood samples from 12 animals that had no antibodies against this piroplasm in serum tested by ELISA. These are probably cases of recent infection in which there was not enough time to allow the animals to mount an effective immune response with the production of detectable levels of antibodies to *T. equi*. In this respect, horses experimentally infected with *T. equi* and *B. caballi* take from 3 to 20 days to produce antibodies against these piroplasms.

With regard to *B. caballi* infection, one of the seropositive animals was a donkey originally from the city of Sorocaba, State of Sao Paulo (SP), southeast Brazil, that was relocated to the city of Passo Fundo,

State of Rio Grande do Sul (RS), southern Brazil. *Anocentor nitens*, the vector for *B. caballi*, occurs in pastures in Sorocaba (Martins et al., 2015). Indeed, both *T. equi* and *B. caballi* and their vectors (Machado et al., 2012) are well distributed in the state of Sao Paulo, from where the referred pony was transferred. One pony and three horses from the Brigade, all born and raised in the State of Rio Grande do Sul (RS), southern Brazil, were also seropositive for piroplasms. In our study, a small number of serum samples were positive for *B. caballi* by ELISA. This low number of *B. caballi* positive samples could stem from the fact that tick vectors, such as *A. nitens*, are not present in the studied area

(IPVDF, 2013). Therefore, the search for additional tick vectors and other routes of transmission for *B. caballi* in the state of Rio Grande do Sul is much needed in order to better understand the prevalence and epidemiology of equine piroplasmiasis in the area.

Despite the fact that equine piroplasmiasis is a disease of worldwide importance due to growing international trade of livestock and high intercontinental movement of horses (Nicolaiewsky et al., 2001), information about the epidemiology of this parasitic disease affecting equids in different countries is scarce. In recent years, major advances in diagnostic molecular biology have allowed the identification of several genotypes of *Theileria* and *Babesia* in Equidae (Bhoora et al., 2009; Nagore et al., 2004). In the present study, *T. equi* 18S rRNA gene was PCR-amplified in 24.4% (22/90) of the blood samples tested. Heim et al. (2007), when conducted a study on piroplasmiasis in a population of horses from slaughterhouses in southeast Brazil, found that 59.7% of the blood samples were positive for *T. equi* based on the amplification of a fragment of the *T. equi* EMA-1 gene.

The 18S rRNA gene has been successfully used for the molecular characterization of equine piroplasmids. Besides, new genotypes of equine piroplasmids have been identified in several regions around the world using this approach (Bhoora et al., 2009; Munkhjargal et al., 2013; Qablan et al., 2013). The present study showed that some genotypes clustered with 18S rRNA sequences belonging to other two genotype groups previously reported (Bhoora et al., 2009). Moreover, as opposed to a previous study (Heim et al., 2007) performed in different geographical regions in Brazil in which no sequence variation was detected in the EMA-1 and RAP-1 genes within *T. equi* and *B. caballi*, respectively, the PCR-amplified sequences from equids in the State of Rio Grande do Sul (RS) were grouped into different clusters and groups. The genetic variation found in *T. equi* 18S rRNA sequences in the present study and in other previous studies (Ketter-Ratzon et al., 2017; Ros-García et al., 2013; Salim et al., 2010) could be due to the introduction of infected animals into a herd (Salim et al., 2010) and/or sexual reproduction of piroplasmids in tick vectors (Bhoora et al., 2009).

Herein, phylogenetic analysis showed that *T. equi* genotypes detected in horses in Brazil do not seem to be geographically restricted, since genotypes detected in animals sampled in different states, namely São Paulo and Rio de Janeiro, clustered together.

The high genetic diversity in *T. equi* 18S rRNA sequences (Bhoora et al., 2009; Qablan et al., 2013) suggest the presence of new *Theileria* species in equine populations. However, a recent study evaluated several 18S rRNA sequences positioned in different groups showing that the average divergence of paired sequences between groups (B and E) was 0.01 and therefore shared 99% identity to one another (Ketter-Ratzon et al., 2017). Although it is difficult to determine where genetic diversity ends and a new species starts, a multifactorial approach including 18S rRNA analysis, multilocus sequence typing (MLST), full genome analysis and ecological factors could help delineate new *Theileria* species.

Since the study area is considered a strong market for breeding, raising and buying/selling of horses with frequent relocation/movement of animals between different regions of the country and exportation/importation of animals with new additions into herds, the data generated by this epidemiological survey would be useful for the establishment of preventive measures and implementation of disease control programs where animals are exported to other countries or participate in equestrian events.

5. Conclusion

Serological and molecular diagnostic tests are important tools to study the epidemiology of equine piroplasmiasis. The results of the present survey show that *T. equi* is the main causative agent of equine piroplasmiasis in Passo Fundo, RS, Brazil.

Using a phylogenetic approach, 9 different *T. equi* genotypes were identified for the first time in Equidae in south Brazil. These findings

confirm the hypothesis that a large genetic diversity of piroplasmids exist. However, additional studies must be carried out in order to better understand the importance of such diversity in the epidemiology of equine piroplasmiasis.

Declaration of interest

The authors declare no actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work that could inappropriately influence, or be perceived to influence our work.

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