

Survey of vector-borne and nematode parasites involved in the etiology of anemic syndrome in sheep from Southern Brazil

Pesquisa de hemoparasitos transmitidos por vetores e nematódeos envolvidos na etiologia de síndrome anêmica em ovinos no Sul do Brasil

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Abstract

Although anemia has been historically linked to *Haemonchus contortus* infection, other infectious agents, such as hemotropic mycoplasmas and tick-borne disease pathogens, may also lead to anemic crisis in sheep. This study has aimed to investigate infections related to anemia in a sheep herd from Bandeirantes City, Paraná State, southern Brazil. Seven out of forty-two (16.6%; 95% CI: 8.32–30.6%) sheep were positive for hemoplasmas by a PCR targeting the 16S rRNA gene and all tested negative for *A. marginale*/*A. ovis* and *Babesia/Theileria* spp. by PCR based on *msp4* and 18S rRNA genes, respectively. Two (4.7%; 95% CI: 1.32–15.79%) animals were infested with *Rhipicephalus microplus* ticks. Fecal egg counting was performed in 38 sheep and 24 (63.15%; 95% CI: 47.2–76.6%) presented > 500 eggs per gram. Phylogenetic analysis of partial sequences of the detected hemotropic *Mycoplasma* sp. 16S and 23S rRNA genes confirmed that the animals were infected with *Mycoplasma ovis*. Polymorphism analysis of partial 16S rRNA sequences showed three different genotypes of *M. ovis* infecting sheep assessed in the present study. *Mycoplasma ovis* and gastrointestinal nematodes occurs in sheep from the northern region of Paraná State.

Keywords: Small ruminants, hemoplasmas, *Mycoplasma ovis*, *Anaplasma marginale*, piroplasmids, gastrointestinal nematodes.

Resumo

Embora a principal causa de anemia seja historicamente relacionada à infecção por *Haemonchus contortus*, outros agentes infecciosos, como micoplasmas hemotrópicos e patógenos transmitidos por carrapatos, também podem causar quadros anêmicos em ovinos. O presente estudo objetivou investigar infecções relacionadas à anemia em um rebanho de ovinos, na cidade de Bandeirantes, Estado do Paraná, sul do Brasil. Sete (16,6%; 95% CI: 8,32–30,6%) de 42 ovinos foram positivos para hemoplasmas pela PCR do gene 16S rRNA, enquanto todos foram negativos para *A. marginale*/*A. ovis* e *Babesia/Theileria* spp. por ensaios da PCR baseados nos genes *msp4* e 18S rRNA, respectivamente. Dois (4,7%; 95% CI: 1,32–15,79%) animais estavam infestados por carrapatos *Rhipicephalus microplus*. Dos 38 animais nos quais foi realizada a contagem de ovos por grama de fezes (OPG), 24 (63,15%; 95% CI: 47,2–76,6%) apresentaram valores >500 para OPG. A análise filogenética das sequências parciais dos genes 16S rRNA e 23S rRNA de hemoplasmas confirmou a infecção por *Mycoplasma ovis*. A análise de polimorfismos de um fragmento do gene 16S rRNA mostrou a ocorrência de três genótipos diferentes de *M. ovis* nos animais. *Mycoplasma ovis* e nematódeos gastrointestinais ocorrem em ovinos da região nordeste do Estado do Paraná.

Palavras-chave: Pequenos ruminantes, hemoplasmas, *Mycoplasma ovis*, *Anaplasma marginale*, piroplasmas, nematódeos gastrointestinais.

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Introduction

Brazilian sheep herd has been estimated to comprise more than 18 million animals. The southern region of Brazil is home to 30% of the herd, which are mainly reared for meat production (IBGE, 2016). Anemia is considered a concerning condition for animal health leading to production losses. Gastrointestinal (GI) parasites and vector-borne pathogens (VBP) have been considered an important cause of anemia in sheep worldwide.

The GI parasite *Haemonchus contortus*, which parasitizes the sheep's abomasum and causes acute hemorrhagic anemia, may lead to sudden death and important losses in sheep industry (Lane et al., 2015; Taylor et al., 2007). Although anemia is commonly linked to *H. contortus* infection in sheep, it is noteworthy that other infectious agents, such as hemotropic mycoplasmas (hemoplasmas) and VBP may also lead to anemic crisis (Yeruham et al., 1998; Neimark et al., 2004; Hornok et al., 2009; Alessandra & Santo, 2012). Nevertheless, VBP have not been historically included in the differential diagnosis of anemia by veterinary practitioners in Brazil.

Hemoplasmas are small and pleomorphic bacteria of red blood cells that infect many different species of vertebrate hosts (Messick, 2004). Even though two hemoplasma species have been initially reported, namely *Mycoplasma ovis* (formerly *Eperythrozoon ovis*) and '*Candidatus Mycoplasma haemovis*' (Hornok et al., 2012). The complete genome sequence of *M. ovis* (strain Michigan) revealed two copies of the 16S rDNA genes, which corresponded to the previously reported sequences of *M. ovis* and '*Ca. M. haemovis*' (Deshuillers et al., 2014). In sheep, *M. ovis* infection may cause weight loss, hyperthermia, mucosal pallor, and hemolytic anemia (Neimark et al., 2004; Aktas & Ozubek, 2017; Martínez-Hernández et al., 2019). In Brazil, *M. ovis* has been detected in deer (Grazziotin et al., 2011a, b; André et al., 2020), goats (Machado et al., 2017), and sheep (Souza et al., 2019).

Anaplasma marginale, the causative agent of bovine anaplasmosis, has been detected in goats from the northeastern region of Brazil (Da Silva et al., 2018) and sheep from Iran (Yousefi et al., 2017). However, the epidemiological and clinical effects of *A. marginale* infection in small ruminants remain to be fully established. On the other hand, *Anaplasma ovis* has been reported infecting sheep and goats from different countries, such as Italy (De La Fuente et al., 2005), Hungary (Hornok et al., 2007), Iran (Jalali et al., 2013), China (Zhang et al., 2016; Yang et al., 2018), and Turkey (Aktas & Ozubek, 2018). Although *A. ovis* infection often causes mild pathogenicity in sheep (Hornok et al., 2007; Renneker et al., 2013), its occurrence is highly involved in concurrent infections with different hemoparasite species in sheep (Jalali et al., 2013; Yang et al., 2015; Sevinc et al., 2018; Ringo et al., 2018), which may contribute to aggravating animal clinical conditions (Aktas & Ozubek, 2018). To date *A. ovis* has never been reported infecting small ruminants in Brazil. Despite the high host specificity of *Rhipicephalus microplus* to cattle (Dantas-Torres et al., 2009; Ma et al., 2016), this tick species may be found parasitizing small ruminants (Brito et al., 2005; Da Silva et al., 2018).

A wide range of Piroplasmorida species, mainly from the genera *Theileria* and *Babesia*, may infect sheep. Although piroplasmid infections in small ruminants may be considered negligible when compared to cattle infections, the number of studies regarding these tick-borne agents has raised with the increasing economic interest in these animals (Schnittger et al., 2003; Ozubek & Aktas, 2017). These infections may cause fever, anorexia, mucosal pallor, hemoglobinuria, and anemia in sheep (Yeruham et al., 1998; Hassan et al., 2015). *Rhipicephalus*, *Hyalomma*, and *Haemaphysalis* ticks have been incriminated as putative vectors for these agents (Morzaria, 1998; Tian et al., 2004; Uilenberg, 2006). In Brazil, studies on piroplasmid infection in sheep have not been reported so far.

Co-infection with *M. ovis* and other pathogens, such as GI nematodes and tick-borne pathogens, may lead to hemolytic anemia and reflect on production decay and mortality (Hornok et al., 2009; Abdullah et al., 2013). Although ovine production has a notable importance, only one study on *M. ovis* has been reported in sheep from Brazil (Souza et al., 2019), whereas the occurrence of *A. marginale* and piroplasmids in sheep from this country remains unknown. Accordingly, the present study aimed to investigate the role of VBP and GI nematodes in the occurrence of anemia in a sheep herd from southern Brazil.

Material and Methods

Ethical approval

This study was approved by the Ethics Committee for Animal Experimentation and Animal Welfare of Universidade Federal do Paraná (protocol 030/2019) and conducted according to the ethical principles of animal experimentation, adopted by the Brazilian College of Animal Experimentation.

Study

A total of 42 female sheep from Bandeirantes municipality (23°06'28"S 50°21'36"W), Paraná State, southern Brazil, were evaluated for the presence of hemoplasmas, tick-borne pathogens (*Anaplasma* spp., *Babesia* spp., and *Theileria* spp.), and GI parasite infection. The animals were co-grazed with cattle in a paddock, where tick infestation is common during the entire year.

Sampling

Blood samples (up to 5 mL) were collected from the sheep by venipuncture of the jugular vein in commercial tubes containing EDTA (BD Vacutainer®, Franklin Lakes, NJ, USA). Fecal samples were obtained directly by rectal collection, identified, and stored in isothermal recipients until analysis.

Ticks found on animals were directly removed using a commercial hook (O'TOM Tick Twister®, Lavancia, France), and kept in labeled absolute ethanol-containing tubes until identification according to morphological taxonomic keys (Barros-Battesti et al., 2006).

Evaluation of packed cell volume

The packed cell volume (PCV) was measured by centrifugation (10,000 rpm for five minutes). A PCV value of < 0.27 L/L was used as an indicator of anemia (Weiss et al., 2010). Thereafter, aliquots of blood were stored at -20 °C until molecular testing.

DNA extraction

Isolation of genomic DNA from sheep blood samples was performed using a commercial kit (Illustra™ Blood GenomicPrep Mini Spin Kit, GE Healthcare, Little Chalfont, UK). Nuclease-free water was used as negative control to monitor cross-contamination.

Polymerase Chain Reactions (PCR)

A conventional PCR for the mammalian endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was performed for all samples to monitor the DNA extraction, as previously described (Birkenheuer et al., 2003). Thereafter, DNA samples were tested using a conventional pan-hemoplasma PCR assay targeting a fragment (900 bp) of the 16S rRNA gene of hemoplasmas (Hoelzle et al., 2011; Machado et al., 2017). This assay has been validated for the diagnostic of hemoplasmas and is able to detect 4.32 DNA copies/μL (Machado et al., 2017).

The primer set targeting a fragment of the 23S rRNA gene of hemoplasmas was designed using Primer3 software (Koressaar & Remm, 2007; Untergasser et al., 2012) and commercially synthesized (Integrated DNA Technologies, Coralville, IA, USA). Briefly, the 23S rRNA gene sequences of hemotropic *Mycoplasma* spp. (NR_076982, NR_103993, AB740012, HE613254, NR_121969, NR_103970, NR_076983) available in the GenBank® database were retrieved and aligned using Bioedit v. 7.0.5.3 software (Hall, 1999). Potential target sites for forward primers were manually identified; suitable reverse primers and PCR products were selected using Primer3 software (Koressaar & Remm, 2007; Untergasser et al., 2012). Based on the desired product size (800–1,000 bp), melting temperature, minimal pair complementarity, and minimal pair 3'-complementarity (to avoid primer-dimer or hairpin formation), one reverse primer was selected. To verify the proprieties of each primer, PCR suitability tests were performed using SMS software (Sequence Manipulation Suite, Edmonton, Alberta, Canada). The specificity of the prospective primers was checked in silico by BLASTn analysis (Altschul et al., 1999) in order to determine the identity with the sequences deposited in the GenBank® database. The primer set used to amplify an 800-bp fragment was 23S_HAEMO_F (5'-TGA GGG AAA GAG CCC AGA C-3') and 23S_HAEMO_R (5'-GGA CAG AAT TTA CCT GAC AAG G-3').

For the standardization of 23S rRNA PCR protocol, a PCR mixture containing 1X PCR buffer, 1.5 mM of MgCl₂, 0.2 mM of each dNTP, 0.4 mM of each primer, 2.5 U of Taq DNA Polymerase (Taq® PCR Master Mix Kit, QIAGEN, Hilden, Germany), 5 μL of DNA template, made to a final volume of 25 μL with water. The cycling conditions consisted of denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing over a gradient of temperature (53, 54, 55, 56, and 57 °C) for 30 s, extension at 72 °C for 60 s, final extension at 72 °C for 10 min, and cooling at 4 °C. Additionally, a second gradient of annealing temperature was tested (52, 52.5, 53, 53.5, 54, and 54.5 °C), and the annealing temperature was determined

to be 54 °C. The efficacy of a gradient of primer concentration was also determined (0.4 and 0.2 mM of each primer), and the concentration of 0.2 mM of each primer showed a band with higher intensity. *Mycoplasma haemofelis* DNA obtained from a naturally infected cat (Marcondes et al., 2018) and nuclease-free water were used as positive and negative controls, respectively. The amplified PCR products were subjected to gel electrophoresis on 1.5% agarose gels for 1 h at 100 V, and the gels were stained with SYBR (0.1 µL/mL gel) (SYBR™ Safe, Thermo Scientific, Waltham, MA, USA), and visualized under a 312 nm UV light transilluminator (LTB HE, Loccus do Brasil, São Paulo, BR).

Additionally, sheep DNA blood samples were also tested by PCR assays targeting a fragment (870 bp) of *msp4* gene of *A. ovis/A. marginale* (De La Fuente et al., 2007), and a fragment (551 bp) of the 18S rRNA gene of *Theileria* spp./*Babesia* spp. (Almeida et al., 2012). *Anaplasma marginale* and *Babesia vogeli* DNA obtained from naturally infected cattle (de Souza Ramos et al., 2019) and dogs (Mongruel et al., 2018), respectively, were used as positive controls. Nuclease-free water was used as negative control.

Sequencing

Amplicons obtained from three samples that were positive for hemoplasmas were extracted and purified from the gel by enzymatic purification (ExoSAP-IT™ PCR Product Cleanup Reagent, Thermo Scientific, Waltham, USA), evaluated by spectrophotometry for concentration and purity (NanoDrop™ One Spectrophotometer, Thermo Scientific, Waltham, USA), and sequenced from both directions by the Sanger's method (3500 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). Thereafter, the sequences were subjected to BLASTn analysis (Altschul et al., 1999) for determining the identity with sequences previously deposited in the GenBank® database. The nucleotide sequences of the 16S rRNA and 23S rRNA genes of *M. ovis* amplified in this study were submitted to the GenBank® database (GenBank® accession nos. MN173878-MN173880 and MN169108-MN169110, respectively).

Phylogenetic analysis

The partial sequences of 16S and 23S rRNA genes were subjected to multiple alignment with sequences selected from GenBank® using MAFFT available on the GUIDANCE 2 server (Sela et al., 2015) for each gene. The best-fit model of nucleotide substitution was determined using jModeltest v.2.1.10 (Darriba et al., 2012) and was set as HKY+G based on the Akaike Information Criterion (AIC). Each Bayesian reconstruction was performed in Beast 1.8.0 (Li & Drummond, 2012) with three independent runs of 30 million MCMC steps sampled at every 5,000 trees, 10% of the burn-in. The phylogenetic tree was visualized with FigTree software version 1.4.3 (Rambaut, 2014) and the final layout was done with Inkscape version 0.92.3 (Albert et al., 2018). The 16S rRNA tree was rooted with *Mycoplasma bovis* and *Bacillus subtilis* whereas the 23S rRNA tree was rooted with *Mycoplasma pneumoniae* and *Bacillus subtilis*.

Genotype analysis of hemoplasmas

Mycoplasma ovis 16S rRNA sequences were analyzed to determine the number and diversity of found genotypes, using the DnaSP software version 5.10.1 (Librado & Rozas, 2009).

Parasitological analysis

Fecal samples were analyzed by the eggs per gram (EPG) technique (Gordon & Whitlock, 1939). Animals presenting an EPG > 500 were considered positive (Ueno & Gonçalves, 1998).

Statistical analysis

A non-parametric Mann-Whitney test or a parametric unpaired Student's t test was used to compare the mean PCV values between *M. ovis*-positive and negative sheep and EPG-positive and negative sheep. The Chi-square or Fisher's exact test was used to determine associations between anemia and presence of ticks with positivity to *M. ovis* and between anemia and EPG positive results. Odds ratio (OR), 95% confidence interval and *p* values were calculated for each variable. Results considered significantly different when *p* < 0.05. Data were compiled and analyzed in Epi Info™ software (version 7.1.5, CDC).

Results

The mean PCV for sheep was 0.30 L/L. Nine out of 42 (21.42%; 95% CI: 11.71–35.94%) animals were anemic. A total of 38 sheep were evaluated by EPG. Four out of 42 animals did not present feces at the time of sampling and were not evaluated. Twenty-four (63.15%; 95% CI: 47.2–76.6%) animals presented EPG values > 500 and were considered positive for Strongylida-type eggs. A total of 24 adult tick specimens were collected from 2 out of 42 (4.76%; 95% CI: 1.32–1.57%) animals and all were identified as *R. microplus*.

For all samples, the mammalian-endogenous *gapdh* gene was consistently amplified. Seven out of forty-two (16.6%; 95% CI: 8.32–30.6%) animals were PCR-positive for hemoplasmas. Only one animal that was PCR-positive for hemoplasma was anemic (PCV = 0.17 L/L) and presented an EPG value of 4,550. Five sheep were concomitantly positive for *Mycoplasma* spp. and Strongylida-type eggs. The results are summarized in Table 1. All samples tested negative for *Anaplasma* spp. and *Babesia/Theileria* spp. by PCR assays.

Table 1. Obtained results regarding PCV values, amplification of the *Mycoplasma* spp. 16S rRNA fragment by PCR, EPG values and, tick presence/identification in sheep from the present study.

Variable	<i>Mycoplasma</i> – PCR					EPG					
	+/n	(%)	OR	95% CI	P-value	+/n	(%)	OR	95% CI	P-value	
Anemia	Yes	1/9	11.11	0.5625	0.06-5.39	0.5278	6/9	66.67	1.8824	0.40-8.82	0.4765
	No	6/33	18.18				17/33	51.52			
Presence of Ticks	Yes	1/2	50.00	5.6667	0.31-103.45	0.3089					
	No	6/40	15.00								
EPG	Yes	3/23	13.04	0.5265	0.10-2.90	0.3884					
	No	4/19	21.05								

+ = Number of positive animals; n = number of samples; 95% CI = 95% Confidence Interval; OR = odds ratio. The mean PCV for all hemotropic *Mycoplasma*-PCR positive sheep (0.28 L/L) and EPG positive sheep (0.28 L/L) was not significantly different when compared to *Mycoplasma*-PCR negative sheep (0.29 L/L) and EPG negative sheep (0.29 L/L) ($P > 0.05$). EPG-negative: 32.30, 31.60, 27.40, 29.10, 23.90, 28.10, 27.00, 21.50, 32.40, 32.70, 28.60, 31.60, 30.50, 34.40, 34.30, 29.20, 38.10, 15.00, 34.00

Three hemoplasma 16S rRNA sequences obtained in the present study showed > 99% identity with multiple *M. ovis* sequences previously deposited in GenBank® (accession IDs no. KU98374, KU983745, KU512718, JF931135, KF313922, MH615809). Besides, the obtained three hemoplasma 23S rRNA sequences showed 100% identity with *M. ovis* previously deposited in GenBank® (accession no. NR_121969). The phylogenetic analysis of 16S (Figure 1) and 23S rRNA (Figure 2) genes confirmed that the sampled sheep were infected with *M. ovis*. The genotype analysis based on the 16S rRNA gene showed the presence of three different haplotypes, one for each analyzed sequence. Values obtained on haplotype diversity analysis are h (number of haplotypes): 3; Hd (haplotype diversity): 1,0000; and π (nucleotide diversity per site): 0,00000.

No significant association was found between anemia ($p = 0.5278$), presence of ticks ($p = 0.3089$), EPG values > 500 ($p = 0.38848$) and positivity to *M. ovis*. Also, no significant association was found between anemia ($p = 0.4765$) and EPG values > 500 (Table 2).

Discussion

Worldwide, anemia in small ruminants has been generally linked to GI nematode infections (Kaplan et al., 2004; Adogwa et al., 2005; Di Loria et al., 2009). Likewise, veterinary practitioners in Brazil have not historically included VBP in the differential diagnosis of anemia. Anemia may be more severe when the animal is coinfecting with nematodes and *M. ovis*. Although a previous study reported an outbreak of *M. ovis* infection that caused death of sheep from Argentina (Aguirre et al., 2009), the anemic status related to *M. ovis* infection is generally less severe if sheep are kept under good nutritional conditions and do not have a severe worm burden (Messick, 2004). In the present study, one out of nine anemic sheep (PCV = 0.17 L/L) was infected with *M. ovis* and also presented a high EPG value (4,550). The Strongylida suborder includes important parasites for ovine livestock, such as *H. contortus*, that may

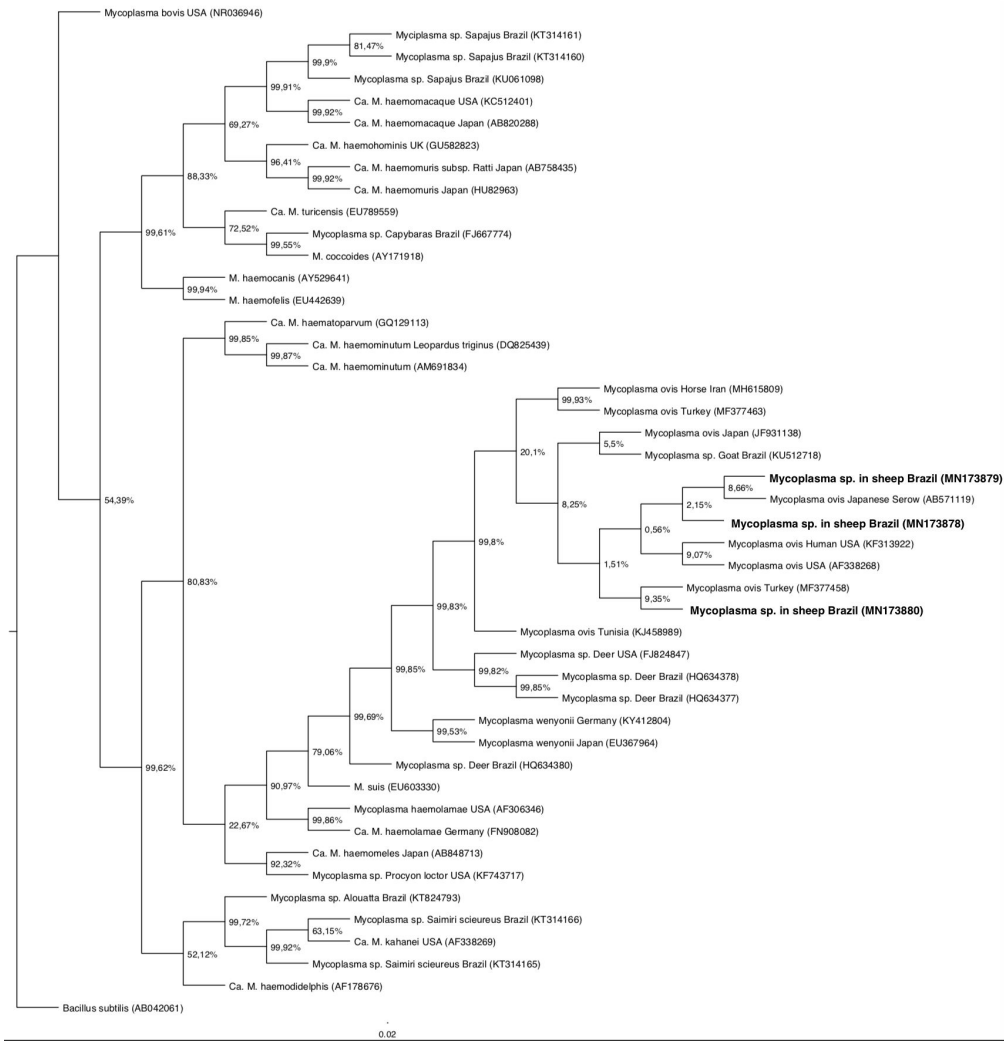


Figure 1. Phylogenetic tree based on partial sequences of the 16S rDNA gene, showing the relationship between the *Mycoplasma* sp. detected in sheep from this study and other hemoplasmas by Bayesian Inference. *Bacillus subtilis* (AB042061) was used as an outgroup. GenBank accession number is after the species name and origin of each bacterium. Analyses were carried out applying the F81+I+G model and 1000 bootstrap replicates for all analyses.

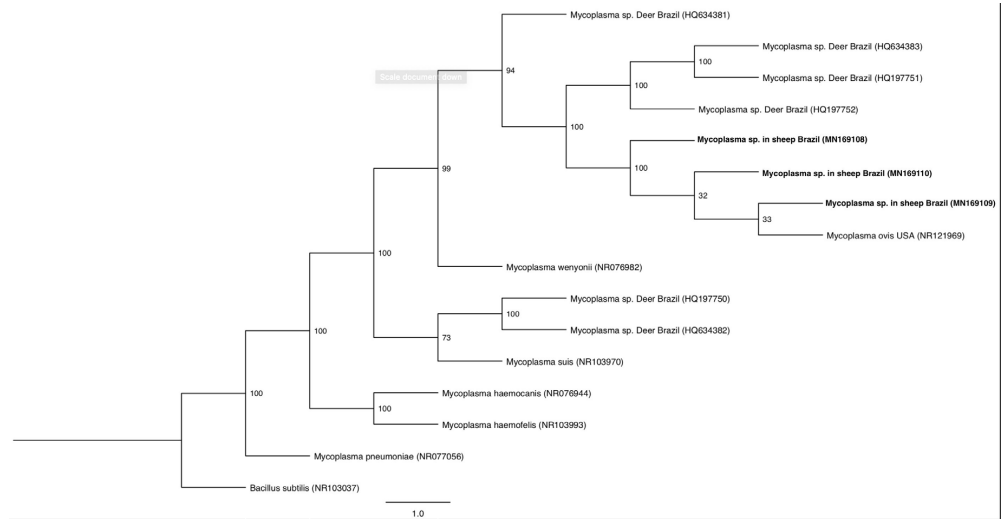


Figure 2. Phylogenetic tree based on partial sequences of the 23S rDNA showing the relationship between the *Mycoplasma* sp. detected in sheep from this study and other hemoplasmas by Bayesian Inference. *Bacillus subtilis* (AB042061) was used as an outgroup. GenBank accession number is after the species name and origin of each bacterium. Analyses were carried out applying the F81+I+G model and 1000 bootstrap replicates for all analyses.

Table 2. Prevalence of *M. ovis*-positive and EPG-positive (>500) sheep within each variable studied, Paraná State, southern Brazil.

Animal identification	PCV (L/L)	<i>Mycoplasma</i> PCR result	Parasitological analysis (EPG)	Presence of <i>R. microplus</i> ticks
1	0.29	+	7,950	
2	0.32	+	250	
3	0.23		Negative	
4	0.28		450	
5	0.27		300	
6	0.31	+	450	
7	0.27	+	Negative	1 male and 10 females
8	0.21		Not collected	
9	0.32		Negative	
10	0.29		1,750	
11	0.32		Negative	
12	0.28		Negative	
13	0.21		5,200	
14	0.31		900	
15	0.19		2,750	
16	0.30		50	
17	0.34		1,550	
18	0.23		3,400	
19	0.34		350	
20	0.34		Not collected	
21	0.33	+	2,000	
22	0.26		3,500	
23	0.29	+	Not collected	
24	0.29		400	
25	0.38		150	
26	0.20		800	1 male and 12 females
27	0.29		1,450	
28	0.31		950	
29	0.29		2,150	
30	0.31		500	
31	0.28		3,400	
32	0.35		1,200	
33	0.33		2,150	
34	0.15		Not collected	
35	0.34		Not collected	
36	0.33		500	
37	0.32		2,950	
38	0.27		4,500	
39	0.28		7,000	
40	0.17	+	4,550	
41	0.29		1,050	
42	0.36		1,700	

EPG = Eggs Per Gram of feces; bold values, PCV values < 0.27 L/L.

lead animals to severe anemic conditions (Amarante, 2014), and thus, may explain the anemic status of the animals studied herein. The remainder eight anemic sheep also presented high values of EPG, ranging from 800 to 5,200.

The present study aimed to investigate infections related to anemia in a sheep herd from southern Brazil. Herein, *M. ovis* was detected in 16.6% of the sampled sheep. Previous studies on the detection of *M. ovis* in Brazil have reported prevalence rates of 39.30% in goats (Machado et al., 2017), 40-87% in deer (Grazziotin et al., 2011a, b; André et al., 2020) and 78.8% in sheep (Souza et al., 2019). Although Souza et al. (2019) detected a higher percentage of positivity to *M. ovis* in sheep from the state of Rio Grande do Sul, divergences on climate conditions, herd management, and employed PCR assays used may explain differences on hemoplasma prevalence rates between studies. Additionally, association between anemia ($p = 0.5278$) or the presence of ticks ($p = 0.3089$) and *M. ovis* infection were not found, similar to that reported in previous studies involving sheep (Rjeibi et al., 2015; Souza et al., 2019) and goats (Machado et al., 2017). Moreover, associations between anemia and EPG values > 500 were not found as well.

It is important to state that there are some limitations on the present study. A convenience sampling was performed in order to investigate infections related to anemia in the studied sheep herd. Although all animals from the herd have been sampled, a low number of samples were provided. Furthermore, conventional PCR assays may present a low sensitivity when compared to quantitative PCR and may lead to false negative results (Willi et al., 2007). Thus, the prevalence found herein may be higher.

Phylogenetic analysis of the 16S and 23S rRNA gene sequences from sheep that were positive for hemoplasmas confirms that animals were infected with *M. ovis*. In the present study, while two *M. ovis* 16S rRNA gene sequences (MN173878 and MN173879) clustered together with *M. ovis* isolated from Japanese serows (*Capricornis crispus*) from Japan (AB571119), the MN173880 sequence clustered together with a *M. ovis* sequence from Turkish sheep (MF377458). The analysis for polymorphisms showed that three different haplotypes of *M. ovis* were infecting sheep in the studied herd. The presence of polymorphisms may cause incongruities in the post-test probability values, which may explain the low values obtained. Additionally, phylogenetic analysis of the *M. ovis* 16S rRNA gene showed marked differences from sequences isolated from the same herd. Genotype diversity of *M. ovis* has been previously reported in flocks from China (Wang et al., 2017) and Mexico (Martínez-Hernández et al., 2019) and, more recently, among deer from Brazil (André et al., 2020). Interestingly, the human-associated *M. ovis* genotypes were more related to genotypes detected in sheep and goats when compared to those found in deer (André et al., 2020). Therefore, future studies aiming to investigate the occurrence of *M. ovis* infection in sheep herd workers are needed.

Even though the 16S rRNA gene has been widely used for phylogenetic analysis, intra-genomic heterogeneities are considered a limiting factor (Rajendhran & Gunasekaran, 2011). In 2014, the genome sequencing of *M. ovis* strain Michigan was reported, showing the presence of two copies of the 16S rRNA gene (Deshuillers et al., 2014). The primers used in the present study cannot specifically target one or another copy from the *M. ovis*-16S rRNA, which may represent unreliable data in sequence analysis.

Regarding the partial 23S rRNA amplification from hemoplasmas, the three sequences amplified in the present study showed 100% identity with *M. ovis* 23S rDNA gene sequence isolated from the USA and deposited in GenBank® (accession no. NR121969). The sequences amplified in the present study clustered together with those of *M. ovis* isolates from sheep from the USA and also formed a large clade with *M. ovis* genotypes detected in deer from Brazil. There are few studies in which the phylogenetic relationship of *M. ovis* has been determined on the basis of 23S rRNA gene assembly. The analysis presented in this study shows similar results with that performed by Grazziotin et al. (2011b), wherein the *M. ovis* deer-related sequences clustered separately from those of the *M. haemofelis* group. Low post-test probability values were also obtained in the 23S rRNA phylogeny of our sequences, which may be related to the genotype diversity among them.

In the present study, all sheep tested negative for *A. ovis/A. marginale* and *Babesia/Theileria* spp. by PCR. Although there are no reports on the detection of *A. ovis* and *A. marginale* or piroplasm infection in sheep from Brazil, *A. marginale* has been detected in goats co-grazed with cattle in the northeastern region of the country (Da Silva et al., 2018). *Anaplasma marginale* is a common tick-borne pathogen that primarily infects cattle from tropical and subtropical areas (Kocan et al., 2010), being transmitted by *R. microplus* ticks in Brazil (Kessler, 2001).

Conclusion

Mycoplasma ovis and GI nematodes occurs in sheep from the northern region of Paraná State and were not related with anemia in the present study. Different genotypes of *M. ovis* occur in sheep from the northern region of Paraná State.

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