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Molecular evidence of Anaplasma phagocytophilum and Theileria equi coinfection in horses from Rio de Janeiro, Brazil

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ABSTRACT

The present study aims to determine the frequencies of *Theileria equi* and *Anaplasma phagocytophilum* antibodies among horses from the state of Rio de Janeiro, Brazil, and to detect the presence of DNA of these pathogens through molecular methods. A total of 98 serum samples of horses from the municipality of Seropedica were tested by indirect immunofluorescence antibody (IFA) to detect anti-*A. phagocytophilum* and anti-*T. equi* IgG antibodies. In addition, quantitative real-time PCR (qPCR) was used to detect these pathogens in the DNA extracted from the whole blood and buffy coat of horses. Bivariate analysis and odds ratio were performed to verify the possible association between positivity and characteristics related to the horses. As evaluated by IFA and qPCR, the frequency of animals that tested positive for *T. equi* was 89.8% (n = 88/98) and 91.8% (n = 90/98), whereas *A. phagocytophilum* was 17.4% (n = 17/98) and 1.0% (n = 1/98), respectively. Serological evidence of exposure to *A. phagocytophilum* and *T. equi* was observed in 16.3% (n = 16/98) of the horses; however, exposure was confirmed by qPCR in only 1.0% (n = 1/98). No statistical association was found in the bivariate and odds ratio analysis. This is the first study reporting the molecular detection of *A. phagocytophilum* DNA in horses from the state of Rio de Janeiro, and also the coinfection of *A. phagocytophilum* and *T. equi* in a horse from Brazil confirmed by molecular methods. Equine granulocytic anaplasmosis is circulating in Brazilian horses, together with *T. equi*, and should be included in the differential diagnosis of tick-borne diseases.

1. Introduction

Most of the Brazilian territory is located in a tropical zone, and a small part is located in a temperate zone. These areas are known to be optimal for the development of ticks. Consequently, several tick species are found in all states parasitizing different hosts, including humans (Dantas Torres, Figueredo & Brandão-Filho, 2006; Serra Freire, 2010). The role of ticks as vectors in many pathogen life cycles, including those of some zoonotic agents, is well established (Jongejan & Uilenberg, 2004).

The tick-borne pathogen *Anaplasma phagocytophilum* is the agent of equine granulocytic anaplasmosis (EGA) and is transmitted by ticks of Ixodidae family. It can cause disease in horses, humans (human granulocytic anaplasmosis), dogs (canine granulocytic anaplasmosis) and other wild and domestic mammals (Stuen, Granquist, & Silaghi, 2013). Horses infected by *A. phagocytophilum* have many debilitating clinical signs, and severe and fatal disease can occasionally occur (Pusterla & Madigan, 2013). Clinical changes including lethargy, inappetence, reduced activity, anorexia, moderate depression, and fever may occur (Pusterla & Madigan, 2013). *Anaplasma phagocytophilum* in horses was first reported in California, and later, it was recognized in many countries in Europe and Asia, where it is endemic. Serological studies were performed in Brazil to verify the presence of anti-*A. phagocytophilum* IgG antibodies. Those reports identified high titers in horses from the states of São Paulo (Salvagni et al., 2010) and Rio de Janeiro (Rolim, Oliveira, & Brasil, 2015), in dogs from Paraná

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(Vieira et al., 2013) and in a deer marsh (*Blastocerus dichotomus)* (Sacchi, Duarte, André, & Machado, 2012). Santos et al. (2013) confirmed the presence of *A. phagocytophilum* in naturally infected dogs and *Ixodes* ticks from the state of Rio de Janeiro through molecular methods.

Several epidemiological studies have reported the circulation of *Theileria equi* in Brazil (Ferreira et al., 2016; Peckle et al., 2013; Vieira et al., 2018). This pathogen is an intraerythrocytic parasite that infects horses and causes equine piroplasmosis, and it is included on the reportable diseases list B from the World Organization for Animal Health (OIE). The presence of *T. equi* has a great impact on the international transit of horses, mainly due to competition purposes because seropositive animals cannot enter in disease-free countries (Friedhoff, Tenter, & Muller, 1990; Knowles, 1996). This parasite is biologically transmitted by ticks from the Ixodidae family (Thompson, 1969) and can also be transmitted iatrogenically through infected blood (Tenter & Friedhoff, 1986), transplacentally (Allsopp, Lewis, & Penzhorn, 2007) and congenitally (Phipps & Otter, 2004; Santos, Santos, & Massard, 2008). The clinical signs are fever, intravascular hemolysis, anemia, jaundice, hemoglobinuria, leg edema, and depression, and in some cases, death may occur (De Wall, 1992). This disease is distributed worldwide and is endemic in many tropical and subtropical regions, including Brazil (Scoles & Ueti, 2015). Using qPCR, Peckle et al. (2013) reported a positivity of 81% of *T. equi* in horses and 7.7% in ticks from the state of Rio de Janeiro.

Considering the high diversity and density of ticks in Rio de Janeiro, the possibility of coinfection with *A. phagocytophilum* and *T. equi* must be considered (Peckle et al., 2013; Pires et al., 2013; Santos et al., 2011). Serological evidence has been previously reported in horses from the Southeast (Prado et al., 2018), Midwest (Salvagni et al., 2010) and Northeast (Nogueira et al., 2017) regions of Brazil. However, the circulation of *A. phagocytophilum* in horses has not been clearly demonstrated by specific molecular methods. Additionally, there are no serological or molecular data regarding *A. phagocytophilum* circulation in horses from Rio de Janeiro.

The aim of the present study was to determine the frequency of *T. equi* and *A. phagocytophilum* in horses from the state of Rio de Janeiro, Brazil, through serological and molecular methods.

2. Materials and methods

2.1. Study and sample collection

This study was performed in the municipality of Seropedica in the state of Rio de Janeiro, Southeast Brazil. A nonprobability sampling method was used.

Whole blood samples from 98 horses (46 females and 52 males), aged from 1.5 to 20 years, were collected by jugular venipuncture and placed in sterile tubes with and without ethylenediaminetetraacetic acid (EDTA). Subsequently, aliquots of whole blood were separated for the deoxyribonucleic acid (DNA) extraction and molecular detection of *T. equi*. In addition, the tubes with EDTA were centrifuged at 2500 × g for 5 min, and the buffy coat was separated for the DNA extraction and molecular detection of *A. phagocytophilum*. Tubes without EDTA were centrifuged at 6000 × g for 10 min, and serum samples were stored at –20 °C until serological analysis.

The ears, head, neck, pectoral, armpit, inguinal and tail areas of the horses were inspected visually to identify the animals infested by ticks. Adult ticks and immature stage were collected and stored in isopropyl alcohol for identification using an appropriate dichotomous key (Barros-Battesti et al., 2006; Martins, Onofrio, Barros-Battesti, & Labruna, 2010).

2.2. Theileria equi and Anaplasma phagocytophilum serology

An indirect immunofluorescence antibody (IFA) test was performed using slides with partially purified antigen from the Jaboticabal strain of *T. equi* (GenBank accession nr. AF255730) that was previously prepared according to the methods described by Baldani, Machado, Raso, and Pinto (2007). To perform the test, the slides were incubated with each serum and diluted at 1:80 in a humid chamber at 37 °C for 45 min. After being washed three times in phosphate-buffered saline (PBS), the slides were incubated with fluorescein isothiocyanate-conjugated equine anti-immunoglobulin G (Sigma-Aldrich®, St. Louis, Mo, USA) diluted at 1:32 and examined under a fluorescence microscope (Hund Wetzlar, model H600/12, Germany) at 400-fold magnification. Serum samples with titers ≥80 were considered positives. The positive control used was from a horse experimentally infected with the Jaboticabal strain of *T. equi*. The negative control used was from a horse free of *T. equi* and *Babesia caballi* infection.

All samples were also tested for IgG anti-*A. phagocytophilum* using an IFA kit (Fuller Laboratories®, USA) following the manufacturer's protocols. All samples were tested at a 1:80 dilution in PBS at pH 7.2. Slide examination was performed using a fluorescence microscope (Hund Wetzlar, model H600/12, Germany) at 400-fold magnification. The positive and negative controls were both supplied by the kit. Serum samples with titers ≥80 were considered positives.

2.3. DNA extraction

DNA was extracted from 100 µL of whole blood and buffy coat using the DNeasy Blood & Tissue Kit (Qiagen®, CA, USA), according to the manufacturer's recommendations. DNA concentrations were determined using the NanoDrop ND-2000 spectrometer (Wilmington, DE, USA), and the DNA samples were subsequently diluted to obtain a final concentration of 30 ng/µL.

2.4. TaqMan real-time PCR assay

2.4.1. Molecular detection of Theileria equi

To detect *T. equi* DNA using the TaqMan PCR system, the Real-Time StepOne Plus® instrument was used to amplify the 81-bp fragment of the *18S rRNA* (Kim et al., 2008). It was used the Be18SF (5′-GCGGTG TTTCGGTGATTCATA-3′) and Be18SR (5′-TGATAGGTCAGAAACT TGA ATGATACATC-3′) primer set and a fluorescent hydrolysis probe, Be18SP (5′-AAATTAGCGAATCGCATGGCTT-3′), which was labeled at the 5′ end with the reporter dye 6-carboxyfluorescein and at the 3′ end with the quencher dye 6-carboxy-tetramethylrhodamine (Kim et al., 2008). The reactions were performed in duplicate on different plates with a final volume of 12 µL, which contained the following reagents: 1X TaqMan® Universal PCR Master Mix, 450 nM of each primer, 250 nM of the hydrolysis probe, an internal exogenous control (TaqMan® Exogenous Internal Positive Control Reagents VIC™ Probe) and 90 ng of total DNA. The thermocycling conditions were 50 °C for 2 min, 95 °C for 10 min, and 45 cycles at 95 °C for 20 s, followed by 55 °C for 1 min.

Whole blood DNA from a horse experimentally infected with the Jaboticabal strain of *T. equi* was used as a positive control. Two positive controls with approximately 1×10^4 and 10 copies of the *18S rDNA,* one negative control from a naïve horse (never exposed to ticks) and a blank control (Nuclease-Free Water, Ambion®) were added to each reaction plate.

The analytical sensitivity and efficiency (97.7%) of the assay were determined in this study, showing a range of 3×10^4 to 3 copies using the methods described by Peckle et al. (2013). The specificity was tested by Kim et al. (2008), who reported no amplification when using positive controls of *B. caballi* and *Trypanosoma evansi*.

The quantification cycle (Cq) was standardized between plates and was manually allocated three cycles after the fluorescence base. Samples with Cq values less than or equal to 40 cycles were considered positive.

2.4.2. Molecular detection of Anaplasma phagocytophilum

DNA samples obtained from each horse's buffy coat were analyzed using the TaqMan PCR system to amplify the 122-bp fragment of the *msp2* gene of *A. phagocytophilum*. It was used the 903F (5′-AGTTTGACT GGAACA CACCTGATC-3′) and 1024R (5′-CTCGTAACCAATCTCAAGCT CAAC-3′) primer set and a fluorescent hydrolysis probe, *939p-* (5′-TTA AGGACAACATGCTTGTAGCT ATGGAAGGCA-3′) (Drazenovich, Foley, & Brown, 2006). The reactions were performed in triplicate on different plates with a final volume of 12 µL, which contained the following reagents: 1X TaqMan® Universal PCR Master Mix, 2 pmol of each primer, 100 pmol of the hydrolysis probe, an internal exogenous control (TaqMan® Exogenous Internal Positive Control Reagents VIC™ Probe); and 90 ng of total DNA. The thermocycling conditions were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s, followed by 60 °C for 1 min. The genomic DNA from *A. phagocytophilum* used as positive control was obtained from the slides of the IFA kit, and ultra-pure water was used as a negative control.

The Cq was standardized between plates and was manually allocated three cycles after the fluorescence base. Samples with Cq values less than or equal to 40 cycles were considered positive.

The analytical sensitivity and efficiency of the assay were determined in this study with a range of 1×10^6 to 1 copy according to the methods described by Drazenovich et al. (2006). In the same study, the specificity was evaluated using DNA from related organisms and other pathogens transmitted by ticks. Santos et al. (2013) also evaluated the specificity of the primers using DNA from *Anaplasma platys, Neorickettsia risticii, Babesia vogeli, Hepatozoon canis*, and *T. equi*.

2.6. Statistical analysis

The frequency of horses positive for *A. phagocytophilum* and *T. equi,* as identified by IFA and qPCR, was calculated. The IFA and qPCR tests were compared using 2 × 2 contingency tables, and discordant pairs were evaluated using the McNemar nonparametric test, permitting 5% error, with BioStat 5.0 software (Ayres, Ayres Júnior, Ayres, & Santos, 2007).

3. Results

Anti-*A. phagocytophilum* and anti-*T. equi* antibodies were detected in 17.3% (n = 17/98) and 89.8% (n = 88/98) of the horses tested with IFA, respectively. *Anaplasma phagocytophilum* and *T. equi* DNA was detected in 1.0% (n = 1/98) and 91.8% (n = 90/98) of those tested with qPCR, respectively (Table 1).

Serological evidence of simultaneous exposure to *A. phagocytophilum* and *T. equi* was observed in 18.2% (n = 16/98) of the horses examined (Table 1). However, coinfection with *T. equi* and *A. phagocytophilum* was confirmed in only 1.0% (n = 1/98) of the horses tested with qPCR.

The horse that tested positive to *A. phagocytophilum* and coinfected with *T. equi* was a 10-years-old male grade horse. This horse presented clinical signs of *T. equi* and *A. phagocytophilum* infection, with pale mucosa, cachexia and fever. At the time of blood collection, this horse was highly infested by *Amblyomma sculptum*.

Ticks collected from parasitized horses were identified as *A. sculptum* and *Dermacentor (Anocentor) nitens*. Of the horses examined, 81.6% (n = 80/98) exhibited infestation by ticks. Among these, 19.4% (n = 19/98) were infested only by *A. sculptum* and 62.2% (n = 61/98) exhibited infestation by *A. sculptum* and *D. nitens*. There was no infestation exclusively by *D. nitens*.

No statistical association ($p > 0.05$) was found between positivity for *T. equi* or *A. phagocytophilum* and the variables: tick infestation, gender, breed, and age of horses (Tables 2 and 3). However, it is important to emphasize that horses which exhibited tick infestation showed a higher frequency of seropositivity for both agents than those that were not infested by ticks.

Table 1
Frequency of *Theileria equi* and *Anaplasma phagocytophilum* by indirect fluorescent antibody (IFA) testing and real-time PCR in horses in Rio de Janeiro, Brazil.

Test		IFA Anaplasma phagocytophilum		
		Positive	Negative	Total
IFA *Theileria equi*	Positive	16	72	88 (89.8%)
	Negative	1	9	10 (10.2%)
	Total	17 (17.3%)	81 (82.7%)	98 (100%)
Test		qPCR *Anaplasma phagocytophilum* (*msp2* gene)		
		Positive	Negative	Total
qPCR *Theileria equi* (18S rRNA gene)	Positive	1	89	90 (91.8%)
	Negative	0	8	8 (8.2%)
	Total	1 (1.0%)	97 (99.0%)	98 (100%)

4. Discussion

The frequency of positive horses for *T. equi* found in this study is in accordance with previous reports that characterize this region of Brazil as endemic to equine theileriosis (Peckle et al., 2013; Santos et al., 2011). The high frequency of positive horses for *T. equi* occurs because this protozoan is resistant to drugs which difficult the elimination of the pathogen. Thus, mostly of the infected horses remain carriers throughout life (De Wall, 1992; Friedhoff, 1988).

The present study reveals moderate frequency (17.3%; n = 17/98) of seropositive horses for *A. phagocytophylum*. This is an interesting result since the circulation of this bacterium has already been confirmed through molecular techniques using dog blood samples from the municipality of Seropedica, Rio de Janeiro (Santos et al., 2011). Previous studies reported that anti-*A. phagocytophilum* IgG antibodies persist for approximately 300 days after experimental infection (Nyindo, Ristic, Lewis, Huxsoll, & Stephenson, 1978) and may persist up to two years after natural infection (Sellon & Long, 2007). It is noteworthy that only one horse in the present study showed clinical signs suggestive of EGA, which may explain the lower frequency of positive animals.

In Brazil, there have been few serological and molecular studies related to *A. phagocytophilum* in domestic and wild animals. Anti-*A. phagocytophilum* antibodies were detected in 65% (n = 13) of horses in the Brazilian Army (1st Guards Cavalry Regiment, Independence Dragoons, Brasilia - DF, Brazil) and in Goiania county, GO, Brazil, with clinical signs suggestive of EGA (Salvagni et al., 2010). Anti-*A. phagocytophilum* antibodies were also detected in horses from the Southeast (Prado et al., 2018; Rolim et al., 2015), Northeast (Nogueira et al., 2017) and South (Vieira et al., 2013) of Brazil. In addition, Sacchi et al. (2012) detected anti-*A. phagocytophilum* in a marsh deer (*B. dichotomus*).

The frequency of *A. phagocytophilum*-seropositive horses observed in the present study was similar to that found in other countries, such as in the USA (Madigan, Hietala, Chalmers, & Derock, 1990) with 10.4%, Switzerland with 16.7% (Egenvall et al., 2001), France with 11.3% (Leblond et al., 2005), Guatemala with 13% (Teglas et al., 2005), Italy with 17.0% (Passamonti et al., 2010), Denmark with 29% (Hansen, Christoffersen, Thuesen, Petersen, & Bojesen, 2010) and Portugal with 9.3% (Ribeiro, Cardoso, Maia, Coutinho, & Cotovio, 2013). The differences observed in these studies might be associated with the number of animals examined, the serological test used, the cut-off point used to identify positive animals and the degree of exposure to tick vectors in different geographical areas.

Despite the moderate exposure to *A. phagocytophylum* demonstrated by the IFA method, only one horse presented the DNA of this pathogen in the blood at the time of the study. Since it is uncommon for *A. phagocytophilum*-infected horses becoming carriers, it is assumed that the positive horses for IFA test and negative for qPCR were not infected at the time of sample collection (Nyindo et al., 1978; Passamonti et al.,

Table 2
Association of breed, gender, age and ticks infestation with the frequency of *Theileria equi* obtained through of the indirect fluorescent antibody (IFA) testing and real-time PCR assay in horses in Rio de Janeiro, Brazil.

Variables related to the horses	IFA *Theileria equi*					Real-time PCR *Theileria equi*			
	N	Positives (%)	*P value*	OR	CI (95%)	Positives (%)	*P value*	OR	IC (95%)
Gender									
Female	44	93.2	0.51	0.49	0.12–2.02	95.5	0.18	0.27	0.06–1.36
Male	54	87.0				85.2			
Breed									
Defined breed	59	91.5	0.72	0.63	0.17–2.34	88.1	0.74	1.62	0.39–6.67
Mixed breed	39	87.2				92.3			
Age (years)									
< 1	12	100.0	0.46	–	–	100.0	0.46	–	–
1 ≤ 5	17	88.2				88.2			
> 5	69	88.4				88.4			
Ticks infestation									
Absence	18	88.9	0.89	1.13	0.22–5.81	77.8	0.06	3.52	0.88–14.12
Presence	80	90.0				92.5			

N = number of horses sampled, OR = Odds Ratio, CI = Confidence Interval.

Table 3
Association of breed, gender, age and ticks infestation with the frequency of *Anaplasma phagocytophilum* obtained through of the indirect fluorescent antibody (IFA) testing in horses in Rio de Janeiro, Brazil.

Variables related to the horses	IFA *Anaplasma phagocytophilum*				
	N	Positives (%)	*P value*	OR	CI (95%)
Gender					
Female	44	15.9	0.88	0.92	0.31–2.77
Male	54	14.8			
Breed					
Defined breed	59	11.9	0.24	1.92	0.63–5.80
Mixed breed	39	20.5			
Age (years)					
< 1	12	0.0	0.22	–	–
1 ≤ 5	17	11.8			
> 5	69	18.8			
Ticks infestation					
Absence	18	11.1	0.58	1.55	0.32-7.58
Presence	80	16.3			

N = number of horses sampled, OR = Odds Ratio, CI = Confidence Interval.

2010). Some studies have reported that *A. phagocytophilum* DNA in horses after experimental infection can be detected over a period of approximately 4 months in the blood (Franzén et al., 2009) and 38 days in tissues (Chang et al., 1998).

Seropositive animals coinfected with *A. phagocytophilum* and *T. equi* have been reported in Portugal (Ribeiro et al., 2013) and Italy (Laus et al., 2013; Passamonti et al., 2010), with molecular confirmation performed only in the latter report. In Brazil, its serological evidence was observed in horses from the Brazilian Army in the state of Goias, Midwest region of Brazil (Salvagni et al., 2010); however, the molecular evidence was not observed. In the present study, serological evidence of both *A. phagocytophilum* and *T. equi* exposure was detected in 18.2% (n = 16/98) of the horses tested by IFA; however, only 1.0% (n = 1/98) of horses tested by qPCR was positive for *A. phagocytophilum* and *T. equi* at the same time. The present study is the first report in Brazil of the coinfection with *A. phagocytophilum* and *T. equi* in horses, proven by molecular methods.

In Brazil, the vector of *A. phagocytophilum* was not described yet. *Amblyomma sculptum* and *D. nitens* were the tick species identified as parasitizing horses in the present study. In a study performed by Santos et al. (2013), in the same geographical area as the present study, the infestation by *Amblyomma* sp. ticks in dogs had a strong association (OR = 6.12; CI: 2.11–28.15) with *A. phagocytophilum* infection. It is noteworthy that *A. sculptum* was the most frequently observed species (50%, n = 12/24). In this same study, the authors suggested that *Amblyomma* ticks were possible vectors of *A. phagocytophilum* among dogs in Brazil. Several studies have reported that *A. sculptum, D. nitens* and *R. microplus* regularly infest horses in Brazil (Borges & Leite, 1998).

No association (p < 0.05) from gender, breed, age and tick's infestation with the horse's positivity for *T. equi* and *A. phagocytophilum* was observed. The tick infestation (OR = 2.6; CI:1.1–6.2) and the presence of *A. sculptum* on the animals (OR = 4.1; CI: 1.8–9.1) was associated with the presence of *T. equi* DNA in the horses (Peckle et al., 2013). On the other hand, epidemiological studies with *A. phagocytophilum* in horses are lacking in literature, especially in Brazil. Therefore, a greater number of equines are in need to identify factors associated with *A. phagocytophilum* infection. In addition, future studies should be done to prove that ticks are playing an important role in the spread of the disease.

The use of the *msp2* gene target improves specificity because it is not present in some distantly related bacteria and has been shown to be an excellent target for PCR detection. However, there are few molecular studies involving the *msp2* gene of *A. phagocytophilum* in Brazil, which restricts comparisons with other isolates from different animal species, including humans.

5. Conclusions

Molecular evidence of *A. phagocytophilum* and *T. equi* coinfecting a horse in Brazil was confirmed for the first time by the detection of DNA from both agents. The consequence of coinfection with vector-borne pathogens in horses in Brazil needs to be clarified to increase the accuracy in diagnosis, to maintain the epidemiological surveillance of zoonotic infectious agents, and thus apply measures of control and prevention of diseases. In this sense, it is important to include EGA in the differential diagnosis of tick-borne diseases in Brazilian horses. Also, future studies should evaluate other genes to identify vectors of *A. phagocytophilum* and genetic variants that infect horses in Brazil.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Ethics statement

All procedures were performed according to the ethical guidelines for the use of animal samples as permitted by the Research Ethics Committee of the Federal Rural University of Rio de Janeiro (COMEP / UFRRJ), number 23083.001257 / 2012-53.

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