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MOLECULAR INVESTIGATION OF HEMOTROPIC MYCOPLASMAS IN HUMAN BEINGS, DOGS AND HORSES IN A RURAL SETTLEMENT IN SOUTHERN BRAZIL

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SUMMARY

The aims of this study were to determine the prevalence of hemoplasmas in a rural Brazilian settlement's population of human beings, their dogs and horses, highly exposed to tick bites; to identify the tick species parasitizing dogs and horses, and analyze factors associated with their infection. Blood samples from 132 dogs, 16 horses and 100 humans were screened using a pan-hemoplasma SYBR green real-time PCR assay followed by a species-specific TaqMan real-time PCR. A total of 59/132 (44.7%) dog samples were positive for hemoplasmas (21 *Mycoplasma haemocanis* alone, 12 '*Candidatus* Mycoplasma haematoparvum' alone and 21 both). Only 1/100 (1.0%) human sample was positive by qPCR SYBR green, with no successful amplification of 16S rRNA or 23 rRNA genes despite multiple attempts. All horse samples were negative. Dogs >1 year of age were more likely to be positive for hemoplasmas ($p = 0.0014$). In conclusion, although canine hemoplasma infection was highly prevalent, cross-species hemoplasma transmission was not observed, and therefore may not frequently occur despite overexposure of agents and vectors.

KEYWORDS: Hemoplasma; *Mycoplasma haemocanis;* '*Candidatus* Mycoplasma haematoparvum'; Real-time PCR.

INTRODUCTION

Hemotropic mycoplasmas (hemoplasmas) are small, pleomorphic, non-cultivable bacteria that attach to the surface of red blood cells[16]. Hemoplasma species have been described to infect a wide range of mammals worldwide, including humans[3,6,8,9,10,28,32]. There are two hemoplasma species most commonly infect dogs, *Mycoplasma haemocanis* and 'Candidatus Mycoplasma haematoparvum'[13,15,31]. Further, a novel hemoplasma species related to the *Mycoplasma haemofelis* group was detected in free-roaming dogs from Aboriginal communities in Australia[2]. Experimental evidence suggests that the brown dog tick, *Rhipicephalus sanguineus*, may play a role in the transmission of canine hemoplasmas[23], supported by higher hemoplasma prevalence rates reported in dogs from areas where *R. sanguineus* ticks were endemic[1,19].

Another hemoplasma species, closely related to 'Candidatus Mycoplasma haemobos', was detected infecting two horses from Germany[7]. Recently, a novel hemoplasma species, 'Candidatus Mycoplasma haemohominis' was identified as the putative primary agent infecting an immunocompetent human in England[25]. In addition, other hemoplasmas have been detected in human patients with immunodeficiency conditions (e.g., HIV/AIDS, lupus erythematosus and neoplasia)[5,9,14], and/or co-infected with other infectious agents (e.g. *Bartonella henselae*)[26]. The zoonotic potential is supported by several reports of human infection by various hemoplasma species typically found only in animals; these include *Mycoplasma suis*[11,34], *M. haemofelis* and/or *M. haemocanis*[14,25], and *Mycoplasma ovis*[26].

The role of ticks, the zoonotic potential and the likelihood of dogs to be used as sentinels for human infection on hemoplasma transmission remain to be fully established. Accordingly, the aims of the present study were to determine the prevalence of hemoplasma species in a restricted population of dogs, horses and humans highly exposed to tick bites in a rural settlement from *Paraná* State, southern Brazil, and to analyze factors associated with hemoplasma infection.

MATERIALS AND METHODS

Blood samples: A total of 132 EDTA blood samples from dogs, 16 from horses and 100 from human beings, previously surveyed for other pathogens[29], were included in this study. All samples were stored at -80 °C until molecular procedures were performed.

DNA extraction: DNA was extracted from 200 µL of whole blood

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samples using the Illustra™ blood genomicPrep Mini Spin Kit (GE Healthcare, Chalfont, St. Giles, UK), according to the manufacturer's instructions. Negative control purifications using ultra-pure water were performed in parallel, to monitor cross-contamination in each batch of 30 samples.

PCR assays: A PCR for the housekeeping gene of all animal species, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed to ensure successful DNA extraction, as previously described[4]. Briefly, 5 µL of DNA was used as a template for the amplification, in a total reaction mixture of 25 µL containing 1X PCR buffer (New England Biolabs® Inc., Ipswich, MA, USA), 0.2 mM of each deoxynucleoside triphosphate (dNTP), 0.25 U of *Taq* DNA polymerase (New England Biolabs® Inc.) and 0.1 mM of each primer. After initial denaturation at 94 °C for four min., the amplification consisted of 29 cycles of 45 sec. each at temperatures of 95 °C, 55 °C and 72 °C for denaturation, annealing and extension, respectively, with a final extension at 72 °C for two min.; the samples were kept at 4 °C until analyzed. All samples were initially screened using a universal hemoplasma SYBR green real-time PCR (qPCR) assay, as previously described[33]. Samples with threshold cycle (C_T) value < 32 were considered positive and were then submitted to species-specific TaqMan qPCR assays for detection of *M. haemocanis*, 'Ca. M. haematoparvum' and 'Ca. M. turicensis', as previously described[31,32]. In addition, samples with C_T values ranging from 32 to 35 by the universal hemoplasma SYBR green qPCR assay were considered suspect of being infected, and were also submitted to species-specific TaqMan qPCR. Real-time PCR assays were performed using a 7300 Real-Time PCR System (Applied Biosystems, Life Technologies Corporation, Corporation, Carlsbad, CA, USA). Briefly, the 25 µL reaction-mixtures contained 1x HotStar Taq Buffer (QIAGEN Inc., Valencia, CA, USA), 0.4 mM of ROX (5-carboxy-X-rhodamine; Rox Reference Dye, Invitrogen™, Life Technologies™, Carlsbad, CA), 3.0 mM of $MgCl_2$, 0.2 mM of each primer, 0.1 mM of probe, and 5 µL of DNA template. The cycling comprised 95 °C for 15 min., followed by 45 cycles at 95 °C for 10 sec., 60 °C for 30 sec., for *M. haemocanis* and 'Ca. M. haematoparvum', and 45 cycles at 95 °C for 30 sec, 60 °C for 30 sec for '*Ca.* M. turicensis'. Nuclease-free water was used as a negative control, while the recombinant plasmids containing the *M. haemofelis*, '*Ca.* M. haematoparvum' and '*Ca.* M. turicensis' 16S rRNA gene as an insert were used as positive controls. Specificity for each protocol was evaluated using known positive samples for *M. haemofelis*, '*Ca.* M. haemominutum', '*Ca.* M. turicensis', *M. suis*, *M. ovis*, *Mycoplasma haemomuris*, *Mycoplasma coccoides* and *Bartonella henselae*. For absolute quantification, plasmids containing the cloned 16S rRNA gene of *M. haemocanis* and "*Ca.* M. haematoparvum" were generated. Purified DNA was quantified, and serially 10-fold diluted in a solution of 30 µg/mL of salmon sperm DNA (Invitrogen).

Sequencing: The nearly complete 16S rRNA gene and a fragment (1,060 bp) of the 23S rRNA gene from five *M. haemocanis* isolates (10% of positive samples) were sequenced. The 16S rRNA gene of *M. haemocanis* isolates were amplified using a set of previously described primers[9]. Cycling conditions consisted of a two min. denaturation at 95 °C followed by 40 cycles of 95 ºC for 30 sec., 55 ºC for 45 sec., and 68 ºC for 1.5 min. with a final extension of 68 ºC for five min.; the samples were kept at 4 °C until analyzed. A conventional PCR assay for the detection of 23S rRNA gene of *M. haemocanis* strain Illinois (CP003199) was developed based on whole genomic sequence of this organism. The forward primer was manually designed; suitable reverse primers and PCR products were selected using the Primer3 software[22]. The primer set used to amplify a 1,060 bp fragment was Mhc23S-Fw (5'-TAAACAGTCCCCCCTCAT TC-3') and Mhc23S-Rv (5'-AAGTATGAGCCGGCGAGTTA-3'). The PCR mixture for amplification of the 23S rRNA gene contained 2.5 µL of 10x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 0.2 mM of each dNTP (dATP, dGTP, dCTP, dTTP), 10 uM of each primer, 1.25 U of *Taq* DNA polymerase, 5 µL of DNA template made up to 25 µL with water. Cycling conditions consisted of a two min. denaturation at 95 °C followed by 40 cycles of 95 ºC for 1 min., 60 ºC for 45 sec., and 68 ºC for 1.5 min. with a final extension of 68 ºC for five min.; the samples were kept at 4 °C until analyzed. The amplified PCR products were subjected to gel electrophoresis on 1.5% agarose gels for one hour at 100 V, followed by ethidium bromide staining (1 µg/mL), and were viewed under a 312 nm UV light transilluminator. The gels were subsequently photographed using Epi Chem II Darkroom® (UVP, Inc., Upland, California, USA). PCR products were purified from the agarose gel (ZymocleanTM Gel DNA Rec. Kit; Zymo Res. Corp., Orange, CA, USA) and directly sequenced by Sanger method in both directions by Purdue Genomics Core Facility (Purdue University, West Lafayette, IN, USA) using an Applied Biosystems 3730XL sequencer (Applied Biosystems).

The 16S rDNA from five '*Ca.* M. haematoparvum' (1,354 bp) isolates (15% of positive samples) were sequenced as previously described[31]. Multiple attempts to amplify the 16S rRNA and 23 rRNA genes with previous reported PCR assays[7,17,18,30] from the human sample were unsuccessful.

All PCR assays were performed using a commercially available PCR Master Mix kit (New England Biolabs® Inc., Ipswich, MA, USA) and reactions were carried out in an Eppendorf® Mastercycler® gradient thermocycler (Eppendorf Scientific, Inc., Westbury, NY).

Statistical analysis: Either the Chi-square or Fisher's exact test was used to determine the association between the individual factors with hemoplasma infection of the independent variables. Odds ratio (OR), 95% confidence interval and *p* values were calculated separately for each variable. Results were considered significantly different when $p < 0.05$. Data was compiled and analyzed by Epi Info™ Software (version 3.5.3).

RESULTS

Sample prevalence of hemoplasmas: All dogs, horses and human samples consistently amplified the GAPDH gene. When using the universal hemoplasma SYBR green qPCR assay, 59/132 (44.7%; 95% CI, 36-53.6%) dogs were considered positive [threshold cycle (C_T) value < 32]. Among these, 21/59 (35.5%; 95% CI, 24.6-48.3%) were positive for *M. haemocanis* alone, 12/59 (20.3%; 95% CI, 12.0-32.2%) for '*Ca.* M. haematoparvum' alone, 21/59 (35.5%; 95% CI, 24.6-48.3%) co-infected dogs, and all 59 dogs were negative for '*Ca.* M. turicensis'. Five samples that were positive by SYBR green qPCR assay (5/59, 8.4%; 95% CI, 3.6-18.3%) were negative in all species-specific TaqMan qPCR assays performed. In addition, 19/132 (14.4%; 95% CI, 9.4-21.3%) dogs had C_T values ranging from 32 to 35 by the universal hemoplasma SYBR green qPCR assay and were considered suspect of being infected. Among these, only 1/19 (5.3%; 95% CI, 0.9-24.6%) was positive for *M. haemocanis* and '*Ca.* M. haematoparvum' by the species-specific TaqMan qPCR assays, while the remaining 18 (94.9%) were negative in all species-specific TaqMan qPCR assays performed.

All horse samples showed negative results by universal hemoplasma SYBR green qPCR assay (C_T > 35). From the total of human samples analyzed, 1/100 (1%; 95% CI, 0.18-5.45%) exhibited a C_T value of 28 by the SYBR green qPCR assay.

Risk factors for canine hemoplasma infection: Dogs > 1-year-old were more likely to be positive for hemoplasmas than dogs ≤ 1 year of age (OR = 3.63, 95% CI = 1.68-7.83%; p = 0.0014). No significant association was found between gender or presence of ticks, and presence of hemoplasmas. The prevalence of hemoplasmas in dogs within each variable studied is shown in Table 1.

Table 1
Prevalence of hemoplasma in dogs from a rural settlement, *Paraná* State, Southern Brazil according to each variable studied

Variable	+/N (%)	OR	95% CI	*p*-value
Presence of ticks				
Yes	29/73 (39.7%)	0.63	0.31-1.27	0.2013
No	30/59 (50.8%)			
Age (years)				
>1	46/82 (56.1%)	3.63	1.68-7.83	0.0014
≤1	13/50 (26%)			
Gender				
Male	37/83 (44.6%)	0.98	0.48-2.0	0.9715
Female	22/49 (44.9%)			

+, Number of positive animals; N, number of samples per variable; OR, odds ratio; 95% CI, 95% confidence interval.

Hemoplasma blood loads: Mycoplasma haemocanis and '*Ca.* M. haematoparvum' blood loads in the dogs ranged from 1.22 x 10^3 to 1.65 x 10^8 and 2.97 x 10^3 to 1.1 x 10^7 DNA copies/mL of blood, respectively.

Sequencing: The complete 16S rRNA gene of all five *M. haemocanis* isolates analyzed showed 99.9-100% identity with *M. haemocanis* 16S rRNA gene sequences from USA (CP003199, AF197337 and AF407208) and Europe (EF416566, EF416568, GQ129116 and GQ129117). Since *M. haemocanis* 16S rRNA gene sequences analyzed also demonstrated 99% identity with *M. haemofelis* (DQ825458), the 23S rRNA gene from these isolates was also sequenced. All five *M. haemocanis* isolates showed 99.8% identity with *M. haemocanis* 23S rRNA gene sequence (CP003199), and 98.7% identity to *M. haemofelis* 23S rRNA gene sequence (CP002808), supporting the likelihood of *M. haemocanis* infection in all dogs analyzed.

The 16S rRNA gene sequences of all six '*Ca.* M. haematoparvum' showed a 99.8% identity with the 16S rRNA gene sequence of this hemoplasma species from Switzerland (EF416569).

DISCUSSION

To the best of our knowledge, this is the first molecular investigation of hemoplasma infections in a population of dogs, horses and humans that are highly exposed to tick bites in Brazil.

The universal SYBR green qPCR assay used in this study has been shown to amplify a 100 bp fragment of the 16S rRNA gene of ten hemoplasma species and was considered suitable to screen known and even unknown hemoplasma species[33]. Even though this assay has reported sensitivity and specificity of 98.2% and 92.1%, respectively, so that positive results should always be confirmed by species-specific TaqMan qPCR assays or sequencing[33]. In the present study, 59/132 (44.7%) dogs screened by the SYBR green qPCR assay were positive; 54/59 (91.5%) of these confirmed to be infected by species-specific TaqMan qPCR assays. The remaining five dogs positive by the SYBR green qPCR assay were negative in all species-specific TaqMan qPCR assays as well as by universal bacterial conventional PCR assays[7,17]. Further analysis should be conducted to elucidate whether these data represent false-positive results or dogs were infected by a not yet described hemoplasma species that cannot be amplified by the molecular assays applied. Only 1/19 (5.29%) of the dogs in this study with C_T values between 32 and 35 by the SYBR green assay, were confirmed by species-specific qPCR to have a hemoplasma infection. Based on these findings, it was suggested that dog samples with C_T values in this range should be considered suspect of being infected and further evaluated by species-specific TaqMan PCR assays.

High hemoplasma prevalence rates have been previously reported in dogs living in warm climate zones, which may facilitate the maintenance of *R. sanguineus* ticks[1,12,19]. Nevertheless, in the city of Recife, northeastern Brazil, only 0.48% of dogs exposed to ticks, and clinically suspected of having tick-borne diseases, were infected by *M. haemocanis* which was detected by a conventional PCR protocol[20].

An association between hemoplasma infection and presence of ticks in dogs was also not observed (p = 0.2013) in the present study. Our findings corroborated those found in free-roaming dogs from Tanzania[2] and on hospital animals in Central Macedonia[27], which failed to show any association between hemoplasma infection and tick infestation. In the present study, although, at the time of sampling, dog owners reported that 44.7% of the dogs did not have previous contact with ticks, there is likely a negative bias in these data; dogs spent most of their time outside their houses and owners may not have noticed ticks parasitizing their animals. Differences found in the current study may also be attributed to sensitivity of diagnostic tests. Moreover, variable circulating load may impair the association between tick presence and hemoplasma infection on single samplings[2].

We have also found that age (> 1 year of age) was associated with hemoplasma infections (p = 0.0014), contradicting a previous study conducted in dogs from Mediterranean countries that have found that hemoplasma PCR-positive dogs were significantly younger than PCR-negative dogs[19]. Regardless, previous studies have mostly failed to establish an association between infection status and age[2,15,21,27,31]. Differences found may be due to the population type (healthy versus hospitalized animals) or living conditions (rural versus urban areas) of surveyed dogs.

Using the same SYBR green qPCR assay as in the present study, 414 human blood samples from immunocompromised patients from Switzerland and HIV-positive patients from Zimbabwe revealed no positive results[33]. The only positive sample found in our study by the SYBR green qPCR assay (C_T value 28) was from a thirty-four-year-old, avid hunter. He recalled tick and insect bites and kept four dogs at

home; one of these dogs was co-infected by *M. haemocanis* and '*Ca.* M. haematoparvum'.

Hemoplasma infection in horses was only recently reported in two animals from Germany[7]. In that study, horse samples were first screened by the SYBR green qPCR assay, with positive samples submitted to a conventional PCR assay targeting the 16S rRNA gene. Moreover, a prevalence of 26.5% of hemoplasmas in horses originating from one breeding farm in Northern Germany was found[6]. Although our study followed the same methodology, all horse samples tested negative. This could be due to the low sample size and/or type of the tested population; whereas the horses in the present study were considered healthy, the previous study has included clinically ill animals[6,7].

In conclusion, although canine hemoplasma infection and tick bites were highly prevalent, cross-species hemoplasma infection was not observed and therefore may not frequently occur despite overexposure of agents and vectors.

RESUMO

Investigação molecular de espécies de micoplasmas hemotrópicos em cães, equinos e humanos de um assentamento rural do sul do Brasil

Os objetivos deste estudo foram determinar a prevalência de hemoplasmas numa população restrita de cães, equinos e humanos altamente expostos a picadas de carrapatos em assentamento rural brasileiro; identificar as espécies de carrapatos parasitando cães e equinos, e analisar os fatores associados à infecção. Amostras de sangue de 132 cães, 16 cavalos e 100 humanos foram avaliadas utilizando um protocolo pan-hemoplasma em PCR quantitativas em tempo real (qPCR) com SYBR green, seguido de qPCR TaqMan espécie-específicos. Cinquenta e nove/132 (44,7%) cães foram positivos para hemoplasmas (21 *Mycoplasma haemocanis*, 12 '*Candidatus Mycoplasma* haematoparvum' e 21 para ambos). Uma amostra humana do total de 100 (1%) foi positiva pelo qPCR SYBR green, mas os genes 16S rRNA ou 23S rRNA não foram amplificados com sucesso, apesar de inúmeras tentativas. Todas as amostras de cavalos foram negativas. Cães > 1 ano apresentaram mais chance de serem positivos para hemoplasmas ($p = 0{,}0014$). Concluindo, embora infecções por hemoplasmas caninos sejam altamente prevalentes, a transmissão de hemoplasmas entre espécies não foi observada, e desta forma podem não ocorrer de forma frequente apesar da alta exposição aos agentes e vetores.

CONFLICT OF INTEREST STATEMENT

None of the authors had conflicts of interest to declare.

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