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LEONILDA CORREIA DOS SANTOS

MOLECULAR IDENTIFICATION OF HEMOTROPIC MYCOPLASMAS (HEMOPLASMAS) IN WILD MAMMALS

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Thesis presented as partial requirement for the degree of Doctor in Veterinary Science of the Post-Graduation of Veterinary Science, Department of Agricultural Sciences, Federal University of Paraná.

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“The mind that opens to a new idea never returns to its original size”.

Albert Einstein

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RESUMO

Os micoplasmas hemotrópicos (hemoplasmas) são bactérias distribuídas mundialmente que afetam animais domésticos e animais selvagens além dos seres humanos. Eles ainda permanecem incultiváveis in vitro. Os hemoplasmas tem sido descritos como potenciais causadores de anemia hemolítica em mamíferos domésticos e selvagens. O objetivo deste estudo foi detectar por intermédio de métodos moleculares a presença de micoplasmas hemotrópicos em mamíferos selvagens nativos e exóticos. Esta tese de doutorado apresenta três artigos. O primeiro artigo é de revisão sistemática e meta-análise sobre a detecção molecular de hemoplasmas em mamíferos selvagens, utilizando-se os artigos indexados no MEDLINE e SCIELO, no periodo de dezembro de 1967 a outubro 2016. Um total de 45/1235 artigos (3,64%) relacionaram-se à identificação molecular de hemoplasmas em mamíferos selvagens, e 78 espécies de mamíferos selvagens foram relatadas como infectadas. A metanálise foi realizada utilizando um modelo de efeitos aleatórios para comparação dos dados de prevalência disponíveis para mamíferos selvagens em cativeiro e em vida livre e entre as ordens. Uma árvore filogenética com base em sequencias do gene 16S rRNA foi construída, comparada e discutida. Hemoplasmas estão distribuídos em mamíferos selvagens em todo o mundo, com prevalencia de 29,92% (IC 24,53 – 33,74) para todos os animais, sendo 31,00% (IC 24,97 – 37,76, I^2 $p<0,001$) para animais em vida livre e 22,33% (IC 17,20 – 28,47, I^2 $p<0,001$) para animais em cativeiro. O segundo artigo refere-se a pesquisa de micoplasmas hemotrópicos em morcegos, sendo este o primeiro estudo com micoplasmas hemotrópicos em morcegos no Brasil. Foram colhidas amostras de sangue (n = 10) de oito morcegos hematófagos: seis machos, morcego-vampiro comum (*Desmodus rotundus*, família Phyllostomidae), dois machos, morcego-vampiro de patas peludas (*Diphylla ecaudata*, família Phyllostomidae); e duas fêmeas não-hematófagas, morcego-de-Pallas (*Molossus* sp., Família Molossidae), na região de Curitiba, Estado do Paraná, sul do Brasil. Para a anestesia, as gaiola com os morcegos foram colocadas dentro de um recipiente de plástico e o isoflurano foi infundido com uma máquina com oxigênio. A manutenção da sedação foi realizada utilizando máscara de inalação. A punção intracardíaca foi realizada para se obter o sangue, sequencialmente, os morcegos foram submetidos a eutanasia com dose letal de cloreto de potássio intracardíaco. Os esfregaços sanguíneos de dois *Desmodus rotundus*, preparados imediatamente após a colheita de sangue, foram corados com May-Grünwald-Giemsa e examinados por microscopia de luz com ampliação de 1000x para a presença de hemoplasma. O DNA foi extraído de 200 μL de sangue utilizando um kit comercialmente disponível de acordo com as instruções do fabricante. A PCR para o gene desidrogenase gliceraldeído-3-fosfato (GAPDH), foi realizada para garantir a extração bem sucedida do DNA. Em seguida, as amostras foram rastreadas por PCR pan-hemoplasma convencional direcionando para as regiões 16S rDNA específicas para hemoplasmas. Utilizando-se iniciadores universais para o 16S rRNA (Referência), amostras de 2 morcegos da espécie *Desmodus rotundus* que testaram positivo para *Mycoplasma* sp na primeira reação de PCR, foram amplificados. Os produtos de PCR de 745 pb foram purificados a partir do gel de agarose a 1,5% e sequenciados. As sequências nucleotídicas dos isolados de hemoplasmas de morcegos foram submetidas à base de dados GenBank sob o número de acesso KX722541. Foi construída uma árvore filogenética baseada em sequências de genes 16S rRNA. Em geral, 8/10 (80,0%) morcegos testaram positivos para *Mycoplasma* sp. incluindo 5/6 (83,3%) *Desmodus rotundus*, 2/2 (100%) *Diphylla ecaudata* e 1/2 (50,0%) *Molossus* sp. As análises da seqüência parcial do gene 16S rRNA identificaram potencialmente uma nova espécie de hemoplasma infectando morcegos na região de Curitiba, Estado do Paraná, Sul do Brasil. No terceiro artigo, o objetivo do estudo foi aplicar um protocolo PCR de *Mycoplasma ovis* em 12

aoudads (*Ammotragus lervia*) de cativeiro do Zoológico de Curitiba, sul do Brasil. Foi utilizado um total de 12 amostras de sangue com EDTA, previamente pesquisadas para outros patógenos. O DNA foi extraído e um protocolo para PCR do gene desidrogenase gliceraldeído-3-fosfato (GAPDH) foi realizado em todas as amostras para garantir DNA amplificável. Em seguida, todas as amostras foram testadas e resultaram negativas em protocolo de PCR específico para a detecção e amplificação de *M. ovis*. Em anexo estão dois artigos, o primeiro anexo trata-se de uma revisão sobre patógenos em aoudads, com artigos publicados entre setembro de 1959 e outubro de 2016, identificados por meio de busca informatizada nas bases de dados eletrônicas PubMed e SciELO. Alguns patógenos detectados em aoudads, como *Mycobacterium tuberculosis* e *Toxoplasma gondii*, também podem infectar animais domésticos e seres humanos. O segundo anexo refere-se a pesquisa de *Plasmodium* sp. em cervídeos no Brasil. Foi avaliado um rebanho cativo de 22 veado-bororó (*Mazama nana*), quatro veado-mateiro (*Mazama americana*) e seis cervo-do-Pantanal (*Blastocerus dichotomus*) do Sul do Brasil; utilizando microscopia de luz e abordagens moleculares. Os testes microscópico e molecular utilizados não indicaram a presença do parasita nas amostras.

Palavras-chave: Micoplasma Hemotrópico, Hemoplasma, Mamíferos Selvagens, PCR, Patógenos, *Plasmodium* sp.

ABSTRACT

Hemotropic mycoplasmas (hemoplasmas) are worldwide distributed bacteria affecting domestic and wildlife animals besides human beings. They still remain uncultivated in vitro. Hemoplasms have been described as potential causes of hemolytic anemia in domestic and wild mammals. The objective of this study was to detect by molecular methods the presence of hemotropic mycoplasmas in native and exotic wild mammals. This doctoral thesis presents three articles. The first article is of systematic review and meta-analysis on the molecular detection of hemoplasms in wild mammals, using articles indexed in MEDLINE and SCIELO, from December 1967 to October 2016. A total of 45/1235 articles (3.64%) related to molecular identification of hemoplasmas in wild mammals, and 78 wild mammal species were reported to be infected. The meta-analysis was performed using a random-effects model to compare the prevalence data available for wild mammals in capitivity and in free ranging between orders. A phylogenetic tree based on 16S rRNA gene sequences was constructed, compared and discussed. Hemoplasmas are distributed in wild mammals throughout the world, with prevalence of 29.92% (CI 24.53 – 33.74) for all reported animals: 31.00% (CI 24.97 – 37.76, I^2 $p < 0.001$) for wild animals and 22.33% (CI 17.20 – 28.47, I^2 $p < 0.001$) for captive animals. The second article refers to the research of hemotropic mycoplasmas in bats, being this the first study with hemotropic mycoplasmas in bats in Brazil. Blood samples (n=10) were taken from eight hematophagous bats: six males common vampire bat (*Desmodus rotundus*; Family Phyllostomidae), two males hairy-legged vampire bat (*Diphylla ecaudata*; Family Phyllostomidae); and two no-hematophagous females Pallas's mastiff bat (*Molossus* sp.; Family Molossidae), at Curitiba's region, Parana State, southern Brazil. For anesthesia, bat cages were put inside a plastic container and isoflurane was infused with a machine with oxygen. Sedation maintenance was performed using inhalation mask. Intracardiac puncture was performed to obtain blood, sequentially, the bats were euthanized with lethal dose of intracardiac potassium chloride. Blood smears of two *Desmodus rotundus*, prepared immediately after blood collection, were stained with May-Grünwald-Giemsa and examined using light microscopy at 1,000x magnification for the presence of hemoplasma. DNA was extracted from 200 µL blood using a commercially available kit according to the manufacturer's instructions. A PCR for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase GAPDH), was performed to ensure successful DNA extraction. Thereafter, samples were screened by conventional pan-hemoplasma PCR targeting the 16S rDNA regions specific for hemoplasmas. 'Candidatus M. haemovis'-positive goat blood sample and nuclease-free water were used as positive and negative control, respectively. Using universal primers for the 16S rRNA (Reference), samples from two bats of the species *Desmodus rotundus* that tested positive for *Mycoplasma* sp in the first PCR reaction, were amplified. The PCR products of 745 bp were purified from the 1.5% agarose gel and sequenced. The nucleotide sequences of the hemoplasmas isolates from bats were submitted to the GenBank database under the accession number KX722541. A phylogenetic tree based on 16S rRNA gene sequences was constructed. In overall, 8/10 (80.0%) bats tested positive to *Mycoplasma* sp. including 5/6 (83.3%) *Desmodus rotundus*, 2/2 (100%) *Diphylla ecaudata* and 1/2 (50.0%) *Molossus* sp. The analyses of the partial sequence of 16S rRNA gene have identified a potentially novel hemoplasma species infecting bats at Curitiba's region, Parana State, Southern Brazil. In the third article, the objective of the study was to apply a PCR protocol of *Mycoplasma ovis* in 12 captive Barbary sheep (*Ammotragus lervia*) at Curitiba Zoo, in southern Brazil. A total of 12 blood samples with EDTA, previously searched for other pathogens were used. DNA extracted and a PCR protocol for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was performed on all samples to ensure amplifiable

DNA. Subsequently, all the samples were tested and found to be negative using a specific PCR protocol for *M. ovis* detection and amplification. In the supplement are two articles, the first supplement is a review of pathogens in aoudads, with articles published between September 1959 and October 2016, identified through a computerized search in the electronic databases PubMed and SciELO. Some pathogens detected in aoudads, such as *Mycobacterium tuberculosis* and *Toxoplasma gondii*, can also infect domestic animals and humans. The second supplement refers to the research of *Plasmodium* sp. in cervidae in Brazil. A captive herd of 22 deer-bororó (*Mazama nana*), four deer-mateiro (*Mazama americana*) and six marsh deer (*Blastocerus dichotomus*) from southern Brazil were evaluated using light microscopy and molecular approaches. Microscopic and molecular analyses were both negative for parasite presence.

Keywords: Hemotropic Mycoplasma, Hemoplasma, Wild Mammals, PCR, Pathogens, *Plasmodium* sp.

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LIST OF ABREVIATIONS AND SYMBOLS

DNA	Deoxyribonucleic Acid
IUCN	International Union for Conservation of Nature
PCR	Polymerase Chain Reaction
Qpcr	Real-Time Polymerase Chain Reaction
AGID	Agar Gel Immunodiffusion
ELISA	Enzyme Linked Immunosorbent Assay
IFT	Immunofluorescence test
IFAT	Indirect fluorescence antibody test
MAT	Microscopic Agglutination Test
MAT*	Modified direct agglutination test incorporating 2-mercaptoethanol and formalin-fixed tachyzoites
MCAM	Morphometric characteristics of adult male
VNT	Virus neutralization test

SUMMARY

1 INTRODUCTION

Hemotropic mycoplasmas (hemoplasmas) are small pleomorphic bacteria that lacking a cell wall and adhere to the surface of erythrocytes. They still remain uncultivated in vitro (MESSICK, 2004; WILLI et al., 2007c; SYKES, 2010a; TASKER, 2010; WOLF-JÄCKEL et al., 2010, SCHREINER et al., 2012; BARKER & TASKER, 2013; HOELZLE et al, 2014). The genera *Haemobartonella* and *Eperythrozoon*, i.e. the former designation for hemoplasmas, have now been reclassified in the genus *Mycoplasma* based on 16S rRNA genetic analysis and morphological similarities (NEIMARK et al., 2001).

Hemoplasmas are worldwide distributed and may infect a wide variety of mammalian species (MESSICK, 2004; SYKES, 2010a), including companion animals (cats and dogs) (MESSICK, 2004; BIONDO et al., 2009; SYKES, 2010a; TASKER, 2010), livestock (pigs, cattle, sheep and horses) (MESSICK, 2004; BIONDO et al., 2009; DIECKMANN et al., 2010), laboratory animals (mice and monkeys) (NEIMARK et al., 2002; BIONDO et al., 2009) and wild animals (MESSICK, 2004; WILLI et al., 2007a; BIONDO et al., 2009; BONATO et al., 2015; GONÇALVES et al., 2015), as well as human beings occasionally (DOS SANTOS et al., 2008; YUAN et al., 2009; SYKES et al., 2010b; STEER et al., 2011, MAGGI et al., 2013c; MAGGI et al., 2013d).

The transmission routes for hemoplasmas are still unclear; however, experimental transmission has been demonstrated via both oral and parenteral administration of infected blood (BARKER & TASKER, 2013). Arthropod vectors have been suggested to play a role in transmission: ticks, fleas, lice and mosquitoes, such as *Ixodes* sp., *Rhipicephalus sanguineus* (SENEVIRATNA et al., 1973, WILLI et al., 2007b), *Ctenocephalides felis* (SHAW et al., 2004; WOODS et al., 2005) and *Polyplax spinulosa* (HORNOCK et al., 2015), *Polyplax serrata* (BERKAMP & WESCOTT, 1988), wild-caught mosquito (REAGAN et al., 2016).

Hemoplasma infection may vary in clinical presentation, particularly regarding the degree of anemia. The disease may be opportunistic and concurrent with other infectious agents, due to ecosystem disturbance, movement of pathogens or putative vectors and recognition of emerging pathogens (WILLIAMS et al., 2002; WILLI et al., 2007c). Although feline hemoplasmas may induce severe anemia in cats, the clinical spectrum ranges from asymptomatic to life-threatening hemolytic crises, and it is dependent on factors such as the hemoplasma species involved, host susceptibility and whether acute or chronic infections are present (MESSICK, 2004; WILLI et al., 2007c; SYKES, 2010; TASKER, 2010). The consequences of hemoplasm infection in wild animals are still unknown and the clinical

manifestations vary with the type of hemoplasm, the stage of infection, the presence of concomitant diseases and with the immunological conditions of the host (GUIMARÃES, 2014).

The diagnostic techniques for hemoplasmas have historically been based on viewing organisms on erythrocytes in blood smears under a microscope, particularly with Romanovsky-type stain (MESSICK, 2004; TASKER, 2010) and Acridine orange staining (GULLAND et al., 1987; BRUN-HANSEN et al., 1997; STOFFREGEN et al., 2006), followed by use of serological (BALJER et al., 1989; RIKIHISA, et al., 1997; HOELZLE et al., 2007; WOLF-JÄCKEL et al.; 2010; GUIMARÃES et al., 2014) and recently, molecular methods (MESSICK et al, 1998; FOLEY et al., 1998; MESSICK et al., 1999; NEIMARK et al., 2002; GUIMARÃES et al., 2011; VIEIRA et al., 2011). Thus, hemoplasmas were initially detected in several wild mammals through examination of blood smears under an optical microscope, including marsupials (SILVA et al., 2007), common voles (PAWELCZYK et al., 2004), rodents (SILVA et al., 2007, ALSARRAF et al., 2016), squirrel monkeys (CONTAMIN et al, 1999; MICHEL et al., 2000), cynomolgus monkeys (DILLBERGER et al., 1994), opossums (MESSICK et al., 2000), collared peccaries (HANNON et al., 1985), deer (KUTTLER et al., 1967) and llamas (REAGAN et al., 1990; MCLAUGHLIN et al., 1990; FISHER et al., 1996).

1.1 GENERAL PURPOSE

The general purpose of this study was to detect by molecular methods the presence of hemotropic mycoplasmas in native and exotic wild mammals.

1.2 SPECIFIC PURPOSES

Detect and molecularly characterize hemotropic mycoplasmas in native and exotic wild mammals and establish their impact on these species.

To review the literature on the molecular identification of hemotropic mycoplasmas in wild mammals.

This PhD thesis is divided into four main chapters. Chapter 1 is the Introduction containing general information on Hemotropic Mycoplasma. Chapter 2 is a review article entitled “Hemotropic mycoplasma in wild mammals: a systematic review and meta-analysis”,

submitted to the Brazilian Journal of Veterinary Parasitology, ISSN: 1984-2961 Manuscript Number: ID RBPV-2016-0154 (Status: Accepted pending revision). Chapters 3 and 4 are articles entitled “Hemoplasmas (hemotropic mycoplasmas) in bats in southern Brazil” and “Molecular screening for hemotropic mycoplasmas in captive Barbary sheep (*Ammotragus lervia*) in southern Brazil” submitted to the Open Veterinary Journal, ISSN 2218-6050 (Online), ISSN 2226-4485 (Print), Manuscript Number: OVJ-2016-11-086 (Status: Under Editor Evaluation), respectively. Chapter 7 is a Supplement with the articles “Pathogens in aoudads (*Ammotragus lervia*): a review” and “*Plasmodium* sp. entitled “Molecular screening of *Plasmodium* species in captive cervids in southern Brazil”.

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2 HEMOTROPIC MYCOPLASMAS IN WILD MAMMALS: A SYSTEMATIC REVIEW AND META- ANALYSIS

2.1 ABSTRACT

Although hemoplasmas are known to infect domestic mammal species worldwide, the clinical and conservation impacts on wildlife remain to be established. In addition, molecular approaches towards hemoplasmas in wild mammals may provide comparative insights regarding diversity, infection, risk factors, control, treatment and prevention. Accordingly, a systematic review on hemoplasma detection in wild mammals was conducted based on articles indexed in MEDLINE and SCIELO. A meta-analysis was performed using a random-effects model for comparison of the prevalence data available for wild and captive mammals and between orders. A phylogenetic tree based on 16S rRNA gene sequences was constructed, compared and discussed. Hemoplasmas are distributed in wild mammals throughout the world, with prevalence of 29.92% (CI 24.53 – 33.74) for all reported animals: 31.00% (CI 24.97 – 37.76, I^2 $p < 0.001$) for wild animals and 22.33% (CI 17.20 – 28.47, I^2 $p < 0.001$) for captive animals. Since certain hemoplasmas may be considered to have clinical relevance and given the wide range of wild mammal hosts harboring this relatively simple form of life, hemoplasmas show great skill as opportunistic infection agents, along with unique cross-barrier ability for different host species.

Keywords: Hemoplasma, *Mycoplasma*, *Haemobartonella*, *Eperythrozoon*, Hemoparasite, wild mammals.

2.2 INTRODUCTION

Hemotropic mycoplasmas (hemoplasmas) are small pleomorphic bacteria without a cell wall and that adhere to the surface of erythrocytes. The genera *Haemobartonella* and *Eperythrozoon*, i.e. the former designation for hemoplasmas, have been reclassified in the genus *Mycoplasma* based on the analysis of the 16S rRNA gene (NEIMARK et al., 2001).

The transmission routes for hemoplasmas are still unclear; however, oral and parenteral experimental transmissions were demonstrated via administration of infected blood (BARKER & TASKER, 2013). Arthropod vectors have been suggested to play a role in transmission: ticks, fleas, lice and mosquitoes, such as *Ixodes* sp., *Rhipicephalus sanguineus* (SENEVIRATNA et al., 1973, WILLI et al., 2007b), *Ctenocephalides felis* (SHAW et al.,

2004; WOODS et al., 2005), *Polyplax spinulosa* (HORNOCK et al., 2015), *Polyplax serrata* (BERKAMP & WESCOTT, 1988) and wild-caught mosquitos (REAGAN et al., 2016).

Hemoplasmas are worldwide distributed and may infect a wide variety of mammalian species (MESSICK, 2004; SYKES, 2010a), including companion animals (cats and dogs) (MESSICK, 2004; BIONDO et al., 2009; SYKES, 2010a; TASKER, 2010), livestock (pigs, cattle, sheep and horses) (MESSICK, 2004; BIONDO et al., 2009; DIECKMANN et al., 2010), laboratory animals (mice and monkeys) (NEIMARK et al., 2002; BIONDO et al., 2009), wild animals (MESSICK, 2004; WILLI et al., 2007a; BIONDO et al., 2009; BONATO et al., 2015; GONÇALVES et al., 2015) and occasionally human beings (DOS SANTOS et al., 2008; YUAN et al., 2009; SYKES et al., 2010b; STEER et al., 2011, MAGGI et al., 2013c; MAGGI et al., 2013d).

They still remain uncultivated in vitro (MESSICK, 2004; WILLI et al., 2007c; SYKES, 2010a; TASKER, 2010; WOLF-JÄCKEL et al., 2010, SCHREINER et al., 2012; BARKER & TASKER, 2013; HOELZLE et al, 2014), and the laboratory diagnosis have historically been based on microscopic visualization of the organisms on erythrocytes in blood smears, particularly with Romanovsky-type stain (MESSICK, 2004; TASKER, 2010) and Acridine orange staining (GULLAND et al., 1987; BRUN-HANSEN et al., 1997; STOFFREGEN et al., 2006). In addition, the infection has been detected by using serological methods (BALJER et al., 1989; RIKIHISA, et al., 1997; HOELZLE et al., 2007; WOLF-JÄCKEL et al.; 2010; GUIMARAES et al., 2014) and recently, by means of PCR based assays (MESSICK et al, 1998; FOLEY et al., 1998; MESSICK et al., 1999; NEIMARK et al., 2002; GUIMARÃES et al., 2011), the detection has been more sensitive and specific. Thus, hemoplasmas were initially detected in several wild mammals through examination of blood smears under an light microscope, including marsupials (SILVA et al., 2007), common voles (PAWELCZYK et al., 2004), rodents (SILVA et al., 2007, ALSARRAF et al., 2016), squirrel monkeys (CONTAMIN et al, 1999; MICHEL et al., 2000), cynomolgus monkeys (DILLBERGER et al., 1994), opossums (MESSICK et al., 2000), collared peccaries (HANNON et al., 1985), deer (KUTTLER et al., 1967) and llamas (REAGAN et al., 1990; MCLAUGHLIN et al., 1990; FISHER et al., 1996).

Hemoplasma infection may vary in clinical presentation, particularly regarding the degree of anemia. The disease may be opportunistic and concurrent with other infectious agents, due to ecosystem disturbance, movement of pathogens or putative vectors and the recognition of emerging pathogens (WILLIAMS et al., 2002; WILLI et al., 2007c). Although feline hemoplasmas may induce severe anemia in cats, the clinical spectrum ranges from

asymptomatic to life-threatening hemolytic crises, and it is dependent on factors such as the hemoplasma species involved, host susceptibility and whether other acute or chronic infections are present (MESSICK, 2004; WILLI et al., 2007c; SYKES, 2010; TASKER, 2010). The consequences of hemoplasma infection in wild animals are still unknown and the clinical manifestations vary with the type of hemoplasma, the stage of infection, the presence of concomitant diseases and the immunological conditions of the host (GUIMARÃES, 2014).

Accordingly, the aim of the present review was to perform a systematic study on indexed articles relating to molecular detection of hemoplasma in wild mammals, followed by a meta-analysis approach to compare the available prevalence data for free-ranging and captive animals, and between mammal orders. Additionally, a comprehensive phylogenetic tree based on the 16S rRNA gene sequences from hemoplasmas detected in wild mammals was constructed, compared and discussed.

2.3 METHODOLOGY

2.3.1 Data collection

The present descriptive study was conducted through a review of articles published between December 1967 and October 2016, which were identified through a computerized search in the PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and SciELO (http://www.scielo.org/) electronic databases, using the following general descriptors (keywords): hemoplasma, hemotropic mycoplasma, haemoplasma, haemotropic mycoplasma, hemotrophic mycoplasma, haemotrophic mycoplasma, haemomycoplasma, hemoparasite, haemoparasite, *Haemobartonella* wild mammal, *Eperythrozoon* wild mammal and *Mycoplasma* wild mammal. The same descriptors in English were also searched for in the SciELO database, with additional Portuguese and Spanish translations as keywords.

The descriptor search in Medline and SciELO resulted in a total of 1,235 articles. For the present comprehensive systematic review and meta-analysis on hemotropic mycoplasmas in wild animals, the inclusion criteria were based on molecular identification of hemoplasmas in wild mammals. Articles that did not fulfill the inclusion criteria, such as articles on other mycoplasmas and/or on parasitism of domestic species or human beings, articles published in non-indexed journals, articles in which optical microscopy was the sole basis for identifying the hemoplasma genus and species, and articles reporting negative results for hemotropic mycoplasma in wild mammals, were excluded.

2.3.2 Phylogenetic analyses

The 16S rRNA gene sequences of hemoplasmas from wild mammals reported in the articles selected were obtained from the GenBank nucleotide database, with the aim of performing phylogenetic analyses. Sequences smaller than 500 bp were excluded from the analysis, as well as identical hemoplasma sequences detected in the same host species. Thirteen other sequences were also included as references for hemoplasma species (*M. suis* 103930, *M. parvum* AB610850, *M. wenyonii* AF016546, *M. ovis* AF338268, '*Ca*. M. haemominutum' U88564, '*Ca*. M. haematoparvum' AY383241, '*Ca*. M. haemobos' EU367965, *M. haemocanis* AF197337, *M. haemofelis* NR 103953, *M. haemomuris* U82963, '*Ca*. M. haemohominis' GU562823, *M. coccoides* AY171918 and '*Ca*. M. turicensis' DQ157154) and one sequence as an outgroup (*Acholeplasma laidlawii* U14905). Multiple sequence alignments were generated by means of the MUSCLE method (EDGAR, 2004); as a preliminary step, evolutionary distances between sequences were estimated through a pairwise distance matrix, using the *p*-distance method in the MEGA6 software (TAMURA et al., 2013). Sequences in cases in which it was not possible to estimate evolutionary distances and which showed high divergence were excluded from the final dataset. Alignments were performed in Mafft (v7.300b) (KATOH et al., 2005) and were then manually improved in MEGA6 (TAMURA et al., 2013).

The jModeltest 2.1 software (DARRIBA et al., 2012) was used to determine the best nucleotide substitution model, which was determined to be TN93+G+I. Phylogenetic analysis was performed by means of the maximum likelihood (ML) method, using PhyML 3.0 (GUINDON et al., 2010), including 1000 bootstrap replicates. The reconstruction was viewed using FigTree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree) and the final layout was done using Inkscape 0.91 (www.inkscape.org).

2.3.3 Meta-analyses

Following study selection, meta-analysis was applied in an attempt to obtain an integrated value for the frequencies found in these articles (1) for all animals, (2) separately for wild and captive animals and (3) according to animal order. Avoidance of publication bias was achieved through a funnel scatter plot on the ratio between the number of diagnostic chances and the number of samples. Studies dispersed outside space formed by the confidence interval between these parameters were removed. Heterogeneity among the studies was checked using the I-squared test, using a significance level of 5% (Sousa et al., 2009). Finally, the integrated values were estimated by applying a random-effects model. All the analyses

were performed using the "meta" package (SCHWARZER, 2014) in the R environment (R CORE TEAM, 2015).

2.4 RESULTS

2.4.1 Hemoplasma detection

A total of 45/1235 articles (3.64%) related to molecular identification of hemoplasmas in wild mammals, and 78 wild mammal species were reported to be infected. Overall, 44/78 species (56.4%) were described as free-ranging, 22/78 (28.2%) as captive, 7/78 (9.0%) as both free-range and captive and 5/78 (6.4%) as not stated.

From the analysis on articles relating to molecular identification of hemoplasmas in wild animals, tables containing the scientific and common names (WILSON & REEDER, 2005; BONVICINO et al., 2008; IUCN, 2016) of the mammal species and the respective hemoplasmas detected were drawn up and presented (Tables 1, 2, and 3). Table 4 shows Hemoplasma species detected in different wild mammals around the world.

2.4.2 Phylogenetic analysis

The consensus tree, with ML bootstrap values greater than 50% given at each node, is presented in Figure 1. The hemoplasma species name, host and country of origin were reported as cited in the respective article, and the hosts included the orders Didelphimorphia, Primates, Rodentia, Chiroptera, Artiodactyla and Carnivora. The phylogenetic tree revealed two major sister clades composing the haemosuis and the haemofelis subclusters, as expected for hemotropic mycoplasmas (NEIMARK et al., 2001). The hemoplasmas detected in animals of the family Cervidae were clustered in the haemosuis group in a clade comprising *M. wenyonii*, '*Ca*. M. erythrocervae', *M. ovis*, '*Ca*. M. haemocervae' and other new *Mycoplasma* species. One exception (DQ524815) was clustered with '*Ca*. M. haemobos', in the haemofelis group, in a well-supported branch. In the haemosuis group, hemoplasmas detected in animals of the order Artiodactyla were also observed in the *M. suis* and *M. parvum* clade (family Suidae), '*Ca*. M. haemolamae' clade (family Camelidae) and *M. ovis* clade (family Bovidae). Hemoplasmas identified in animals of the order Carnivora formed a cluster containing the clades '*Ca*. M. haemozalophi' (*Zalophus californianus*), '*Ca*. M. haemomeles' (*Meles meles*), '*Ca*. M. haemominutum' (Felidae) and '*Ca*. M. haematoparvum' (Canidae).

The haemofelis cluster included a wide host range, including Rodentia and Chiroptera, which were only found in this group. Hemoplasmas detected in Felidae were grouped in the

M. haemofelis / *M. haemocanis* clade and in the '*Ca*. M. turicensis' clade. One sequence, detected in a leopard cat from Korea (KP843891) (HWANG et al., 2015), was positioned as a sister clade to a mycoplasma detected in a rodent from Brazil (KT215637) (GONÇALVES et al., 2015). A sequence detected in a species of Ursidae (AB725596) (ISO et al, 2013) was also clustered in the *M. haemofelis* / *M. haemocanis* clade. A hemoplasma sequence identified in *Herpestes javanicus* (KJ530704), which was reported as '*Ca*. M. turicensis'-like (SHARIFIYAZDI et al. 2014), clustered as a well- supported basal sister clade to '*Ca*. M. turicensis'. Hemoplasmas detected in non-human primates were positioned in the haemosuis (Cebidae and Atelidae) and haemofelis (Cercopithecidae and Cebidae) subclusters. In both cases, the hemoplasmas species were clustered as sister clades, respectively. Sequences detected in wild animals of Brazil were positioned in both major clades. These sequences were detected in animals of the orders Artiodactyla (haemosuis cluster), Rodentia (haemofelis cluster), Primates and Carnivora (both clades).

Hemotropic mycoplasmas have traditionally been named according to the host in which they were identified (NEIMARK et al., 2001). The analysis of the present study showed that hemoplasmas have a wide host range and geographic distribution. Some species seems to have high cross-species capability. On the other hand, there might be some relationship at family level. Although other genes such as 23S rRNA and RNase P have been used to hemoplasma detection (WILLI et al., 2007a; ANDRÉ et al., 2011; BARKER et al., 2011; GRAZZIOTIN et al., 2011a; GRAZZIOTIN et al., 2011b; VOLOKHOV et al., 2011; KRENGEL et al., 2013; MAGGI et al., 2013b; SASHIDA et al., 2013; TAGAWA et al., 2014), very few were available for use in the phylogenetic analysis. Molecular data have allowed the description of new hemoplasma species or the reclassification of species. Although the 16S rRNA gene is the molecular marker mostly used to report hemoplasma species (MESSICK et al., 2002; NEIMARK et al., 2002, 2001; NEIMARK & KOCAN, 1997; RIKIHISA et al., 1997), additional gene sequences have been proposed to elucidate new species and the taxonomy of the hemoplasmas (DRANCOURT & RAOULT, 2005; HICKS et al., 2014; PETERS et al., 2008; TASKER et al., 2003). Previous studies using 16S rRNA (AQUINO et al., 2014; HIRATA et al., 2012; NEIMARK et al., 2005, 2001; PETERS et al., 2008; TASKER et al., 2003; WILLI et al., 2007, 2006) and other genes, as *rnpB* (PETERS et al., 2008; TASKER et al., 2003), *dnaK* and *gapA* (HICKS et al., 2014), have supported that hemoplasma species form a large clade divided in two subclades. Each of the two subclades, has shown some variation in its topology, when comparing different studies (BONATO et al., 2015; MESSICK et al., 2002; NEIMARK et al., 2001; PETERS et al., 2008), including the

phylogenetic tree presented herein, mainly due to the number of sequences and species used in each analysis, but also due to other variables, as the phylogenetic method used. On the other hand, it was not possible to compare the current analysis with phylogenetic studies comprising hemoplasma sequences from domestic animals or from a specific group of animals, such as felines and canines (AQUINO et al., 2014; HIRATA et al., 2012; TASKER et al., 2003; WILLI et al., 2007, 2006), as they mostly comprised a specific number of hemoplasma species.

2.4.3 Meta-analyses

The free-ranging and captive wild mammals presenting hemoplasmas that were described in the articles evaluated were classified within their order of mammals and then analyzed. There was variability between the integrated values in each group evaluated in the meta-analysis (Table 5). Overall, the prevalence was 29.92% (CI 24.53 – 33.74) for all the animals: 31.00% (CI 24.97 – 37.76) for free-ranging animals and 22.33% (CI 17.20 – 28.47) for captive animals. The evaluation for all the animals according to their orders showed that Carnivores presented significantly lower prevalence than both the other orders and the general prevalence (13.26% and CI 10.58 – 16.48). This was repeated in captive animals, among which Carnivores presented significantly lower prevalence than the other orders (14.82% and CI 10.99 – 19.68).

The heterogeneity tests in all models (all animals, free range, captive and between orders) were significant. These significances showed variability that was not controlled by the statistics tests between the studies; moreover, it did not represent any loss of quality for the values obtained.

2.4.4 Co-infection and other pathogens

The clinical observation that cats coinfected with feline leukemia virus (FeLV) and *Haemobartonella felis* develop more severe anemia than cats infected with *Haemobartonella felis* alone (GEORGE et al., 2002). Statistically significant associations (based on PCR results) were found between FeLV infections with *M. haemofelis* and '*Candidatus* M. turiscensis' in Iberian lynx (MELI et al., 2009). Canine distemper virus (CDV) also has an immunosuppressive effect and secondary infections are common (SYKES, 2010b).

Climate extremes can promote a complex interplay between epidemic and endemic pathogens that are normally tolerated in isolation, but with co-infection, result in catastrophic

mortality. '*Ca* M haemominutum', CDV, *Babesia* sp. and *Hepatozoon* sp. were found in Serengeti lions (*Panthera leo*) (MUNSON et al., 2008).

The presence of the hemotrophic mycoplasma organisms in California sea lions was not accompanied by anemia, even though all hemoplasma-positive animals also were simultaneously infected with filarial nematodes of the genus *Acanthocheilonema* ((VOLOKHOV et al., 2011). The presence of the hemotropic mycoplasma organisms and the accompanying anemias were transient and recurrent within the affected reindeer. Subjectively, the organism load seemed to be correlated with the level of anemia. Although someanimals also were infected with trychostrongyle abomasal nematodes, which likely contributed to their anemic states, an aggressive anthelmintic program was eventually found that eliminated the trichostrongyle abomasal nematodes; however, animals continued to experience bouts of anemia with haemomycoplasma organisms demonstrated in blood smears and by PCR after the nematodes were eliminated from the herd (STOFFREGEN et al., 2006).

Reported of other microorganisms or antibodies detected in animals there were positive for hemoplasma such as viruses, bacteria, protozoa, fungi and nematodes, were also analyzed.

The presence of concurrent antibody response in wild mammals has been observed with viruses such as canine distemper virus (CDV) in cheetahs (KRENGEL et al., 2013), Iberian lynxes (MELI et al., 2009) and in lions (MUNSON et al., 2008); feline leukemia virus (FeLV) in wildcats (WILLI et al., 2007a) and Iberian lynxes (MELI et al., 2009); feline immunodeficiency virus (FIV) in Iberian lynxes (MELI et al., 2009); feline coronavirus (FCoV) in Iberian lynxes (MELI et al., 2009); feline herpesvirus (FHV) in Iberian lynxes (MELI et al., 2009); feline parvovirus (FPV) in Iberian lynxes (MELI et al., 2009); and feline calicivirus (FCV) in Iberian lynxes (MELI et al., 2009).

Bacterial infections included *Anaplasma* sp. in Arctic foxes (MASCARELLI et al., 2015) and leopard cats (HWANG et al., 2015); *Anaplasma bovis* in leopard cats (HWANG et al., 2015); *Anaplasma phagocytophilum* in Iberian lynxes (MELI et al., 2009); *Bartonella henselae* in Iberian lynxes (MELI et al., 2009) and Arctic foxes (MASCARELLI et al., 2015); *Bartonella quintana* in cynomolgus monkeys (MAGGI et al, 2013a); *Chlamydophila felis* in Iberian lynxes (MELI et al., 2009); *Ehrlichia canis* in Arctic foxes (MASCARELLI et al., 2015); *Leptospira* sp. in California sea lions (VOLOKHOV et al., 2011), *Mycobacterium bovis* in Iberian lynxes (MELI et al., 2009); and *Rickettsia felis* in Darwin's foxes (CABELLO et al., 2013).

Protozoa has been reported in relation to *Babesia* sp. in lions (WILLI et al., 2007a; MUNSON et al., 2008); *Hepatozoon* sp. in lions (WILLI et al., 2007a; MUNSON et al., 2008); *Plasmodium falciparum* in squirrel monkeys (NEIMARK et al., 2002) and owl monkeys (BARKER et al., 2011); *Cytauxzoon felis* in Iberian lynxes (WILLI et al., 2007a; MELI et al., 2009); and *Theileria/Cytauxzoon*-like organisms in lions (WILLI et al., 2007a).

Nematodes such as *Acanthocheilonema* sp. in California sea lions (VOLOKHOV et al., 2011) and *Trichostrongylus* sp. in reindeer (STOFFREGEN et al., 2006) has also been reported.

On the other hand, infection with fungus species has only been reported with *Pseudogymnoascus destructans* in little brown bat (MASCARELLI et al., 2014).

2.5 DISCUSSION AND CONCLUSION

Extreme climatic conditions may alter historic host-pathogen relationships and synchronize the temporal and spatial convergence of multiple infectious agents, triggering epidemics with far greater mortality than those due to single pathogens (MUNSON et al., 2008).

Despite the fact that parasites are highly specialized with respect to their hosts, empirical evidence demonstrates that host switching rather than co-speciation is the dominant factor influencing the diversification of host-parasite associations (ARAUJO et al., 2015).

The Stockholm Paradigm postulates that parasite specialists can shift rapidly to novel hosts via Ecological Fitting (EF). EF between hosts and parasites occurs with high enough frequency to influence host range dynamics and the diversity of species and interactions among species (HOBERG & BROOKS, 2015).

In the analysis of the phylogenetic tree it is verified that wild mammals of the Orders: Didelmorphia, Primates, Carnivora and Artiodactila are included in the Haemosuis group and also wild mammals of the Orders: Primates, Rodentia, Chiroptera, Carnivora and Artiodactila are included in Haemofelis group.

Hemoplasma species detected in different wild mammals around the world (Table 5) may be present in different hosts, such as *Mycoplasma haemofelis* for wild felines (*Felis silvestris silvestris, Leopardus pardalis, Leopardus tigrinus, Leopardus wiedii, Lynx lynx, Lynx pardinus, Panthera leo, Panthera tigris, Prionailurus iriomotensis*) and Darwin's fox (*Lycalopex fulvipes*).

Given the wide range of wild mammal hosts harboring this relatively simple form of life, hemoplasmas show great skill as opportunistic infection agents, along with unique cross-barrier ability for different host species.

Continuing research on hemoplasmas in wild mammals may provide comparative insights that fill several pathophysiological gaps such as in relation to their transmission and role in wildlife, livestock and companion animals, as well as in humans. Further studies should be conducted through a multidisciplinary approach, in order to fully establish reliable and affordable methods for diagnosis, treatment, monitoring and prevention of hemoplasma species infections.

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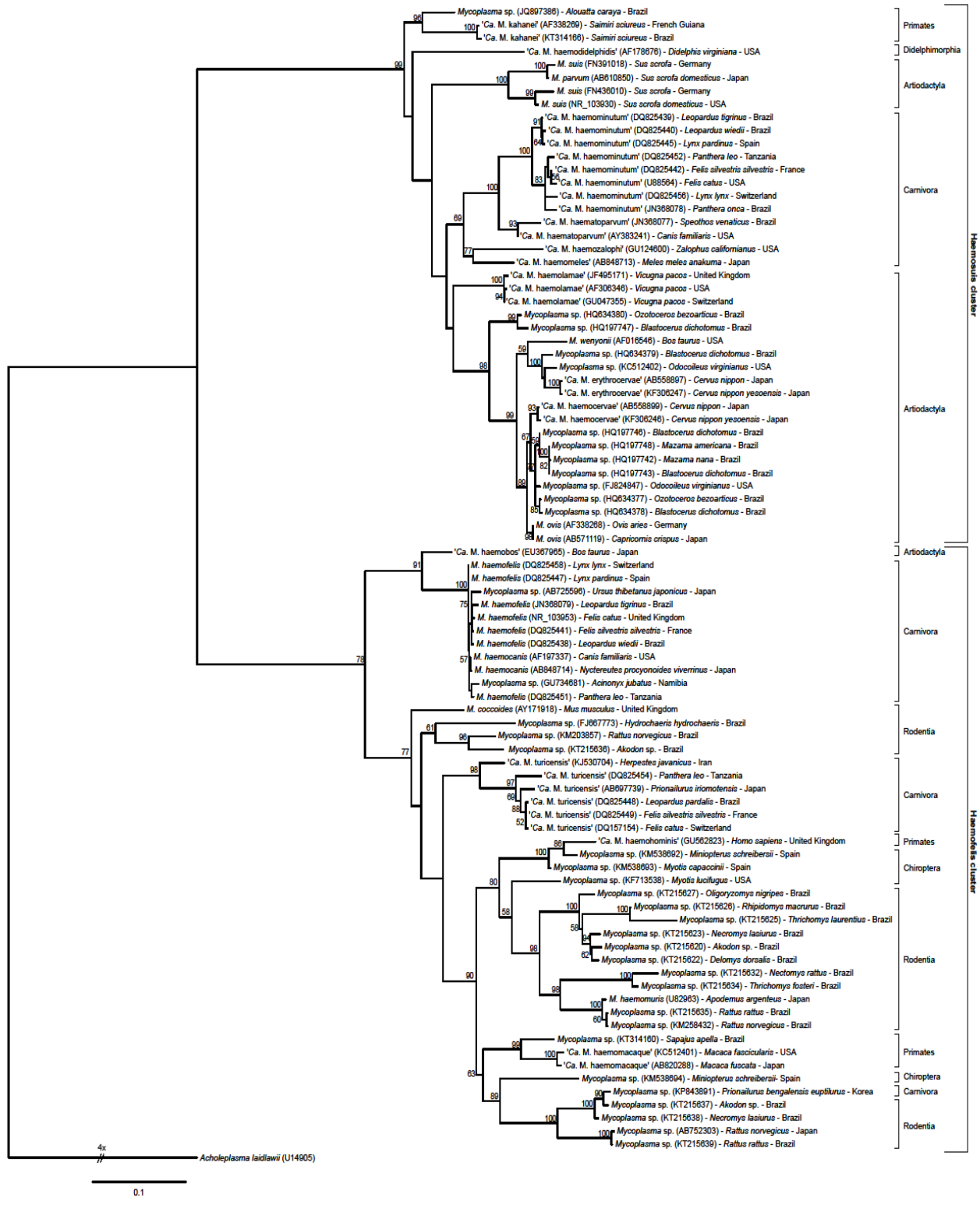

Figure 1. Phylogenetic relationships among 87 hemoplasmas detected in wild mammals, which were inferred from 16S rRNA gene sequences. A maximum likelihood tree was constructed from 1341 aligned characteristics. The data set was resampled 1000 times and bootstrap support values ≥ 50% are indicated at the nodes. Species names are followed by the GenBank accession number, host and country of origin. Host orders are also indicated. The tree was rooted to *Acholeplasma laidlawii* (GenBank U14905). The scale bar represents the expected number of changes per site.

Table 1. Hemoplasma species molecularly detected in wild mammals belonging to the orders Didelphimorphia, Primates, Rodentia and Chiroptera.

Scientific name / Common name	Origin / Status	Number / Prevalence	Hemoplasma species	GenBank / (Reference)
Order: Didelphimorphia				
Didelphis virginiana (opossum)	NI/Sick	1/1 (100%)	'*Ca*. M. haemodidelphidis'	AF178676 (MESSICK et al., 2002)
Order: Primates				
*Alouatta caraya** (black howler monkey)	Free-range/Sick	1/1 (100%)	'*Ca*. M. kahanei', (n) *M*. sp.	JQ897386 (SANTOS et al., 2013)
*Aotus trivirgatus (*owl monkey)	Captive /NI	1/1 (100%)	'*Ca*. M. aoti'	HM123756 (BARKER et al., 2011)
Macaca fascicularis (cynomolgus monkey)	NI/Healthy	44/52 (84.6%)	'*Ca*. M. haemomacaque'	KC512401 (MAGGI et al., 2013a)
Macaca fuscata (Japanese monkey)	Captive /NI	9/9 (100%)	'*Ca*. M. haemomacaque'	AB820288 (SASHIDA et al., 2014)
*Saguinus niger** (black tamarin)	Captive/NI	2/7 (28.6%)	*Mycoplasma* spp., (n) *M*. sp.	NI (BONATO et al., 2015)
Saimiri sciureus (squirrel monkey)	Captive/Healthy	1/1 (100%)	'*Ca*. M. kahanei'	AF338269 (NEIMARK et al., 2002)
*Saimiri sciureus** (squirrel monkey)	Captive/NI	4/16 (25.0%)	'Ca. M. kahanei'	KT314165, KT314166 (BONATO et al., 2015)
Sapajus sp.* (monkey)	Captive/NI	1/1 (100%)	*Mycoplasma* spp., (n) *M*. sp.	KT314161 (BONATO et al., 2015)
*Sapajus apella** (capuchin monkey)	Captive/NI	28/64 (43.7%)	*Mycoplasma* spp., (n) *M*. sp.	KT314160, KT314162, KT314163, KT314164 (BONATO et al., 2015)
Order: Rodentia				
Akodon sp.* (grass mouse)	Free-range/NI	5/27 (18.5%)	*Mycoplasma* spp., (n) *M*. sp.	KT215620, KT215621, KT215636, KT215637 (GONÇALVES et al., 2015)

Scientific name / Common name	**Origin / Status**	**Number / Prevalence**	**Hemoplasma species**	**GenBank / (Reference)**
*Akodon montensis** (montane grass mouse)	Free-range/NI	6/16 (37.5%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
Apodemus argenteus (small Japanese field mouse)	Free-range/Healthy and Sick	NI	*Haemobartonella muris*	NI (RIKIHISA et al., 1997)
Apodemus sp. (field mouse)	Free-range/NI	24/45 (53.3%)	*M. coccoides*	EF175168-EF175170 (WILLI et al., 2007b)
*Calomys sp.** (vesper mouse)	Free-range/NI	4/43 (9.3%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
*Calomys cerqueirae** (Cerqueira's vesper mouse)	Free-range/NI	2/3 (66.7%)	*Mycoplasma* spp., (n) *M*. sp.	NI(GONÇALVES et al., 2015)
*Calomys tener** (delicate vesper mouse)	Free-range/NI	3/10 (30.0%)	*Mycoplasma* spp., (n) *M*. sp.	NI(GONÇALVES et al., 2015)
*Delomys dorsalis** (striped Atlantic forest rat)	Free-range/NI	5/9 (55.5%)	*Mycoplasma* spp., (n) *M*. sp.	KT215622 (GONÇALVES et al., 2015)
*Hydrochaeris hydrochaeris** (capybara)	Free-range/Healthy and Sick	17/21 (80.9%)	*M. coccoides*	FJ667773 (VIEIRA et al., 2009)
*Hydrochaeris hydrochaeris** (capybara)	Captive/Healthy and Sick	3/10 (30.0%)	*M. coccoides*	FJ 667774 (VIEIRA et al., 2009)
*Hydrochaeris hydrochaeris** (capybara)	Free-range and Captive/Healthy and Sick	2/21 (9.5%)	(n) *M*. sp.	NI (VIEIRA et al., 2009)
*Hylaeamys sp.** (wood mouse)	Free-range/NI	3/9 (33.3%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
Myodes glareolus (bank vole)	Free-range/NI	601/880 (68.3%)	*Haemobartonella* sp.	NI (BAJER et al., 2014)

Scientific name / Common name	**Origin / Status**	**Number / Prevalence**	**Hemoplasma species**	**GenBank / (Reference)**
*Mus musculus** (house mouse)	Free-range/NI	1/14 (7.2%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
*Necromys lasiurus** (hairy-tailed bolo mouse)	Free-range/NI	18/51 (35.3%)	*Mycoplasma* spp., (n) *M*. sp.	KT215623, KT215624, KT215633, KT215638 (GONÇALVES et al., 2015)
*Nectomys sp.** (water rat)	Free-range/NI	1/5 (20.0%)	*Mycoplasma* spp., (n) *M*. sp.	KT215631 (GONÇALVES et al., 2015)
*Nectomys rattus** (small-footed bristly mouse)	Free-range/NI	3/8 (37.5%)	*Mycoplasma* spp., (n) *M*. sp.	KT215632 (GONÇALVES et al., 2015)
*Nectomys squamipes** (scaly-footed water rat)	Free-range/NI	2/6 (33.3%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
*Oecomys sp.** (arboreal rice rat)	Free-range/NI	2/6 (33.3%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
*Oligoryzomys sp.** (pigmy rice rat)	Free-range/NI	3/11 (27.3%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
*Oligoryzomys flavescens** (yellow pygmy rice rat)	Free-range/NI	1/3 (33.3%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
*Oligoryzomys fornesi** (Fornes's colilargo)	Free-range/NI	1/2 (50.0%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
*Oligoryzomys nigripes** (black-footed pygmy rice rat)	Free-range/NI	7/24 (29.2%)	*Mycoplasma* spp., (n) *M*. sp.	KT215627-KT215629 (GONÇALVES et al., 2015)
*Oxymycterus sp.** (hocicudo)	Free-range/NI	2/3 (66.7%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
Rattus norvegicus (brown sewer rat)	Free-range/NI	1/9 (11.1%)	(n) *M*. sp.	AB752303 (SASHIDA et al., 2013)
*Rattus norvegicus** (brown sewer rat)	Free-range/ Healthy	31/43 (72.1%)	*M. haemomuris*, (n) *M*. sp.	KM258432, KM 203857, respectively (CONRADO et al., 2015)
*Rattus norvegicus** (brown sewer rat)	Captive/ Healthy	9/20 (45.0%)	*M. haemomuris*, (n) *M*. sp.	KM258432, KM 203857, respectively (CONRADO et al., 2015)

Scientific name / Common name	**Origin / Status**	**Number / Prevalence**	**Hemoplasma species**	**GenBank / (Reference)**
*Rattus rattus** (black rat)	Free-range/NI	14/29 (48.3%)	*Mycoplasma* spp., (n) *M*. sp.	KT215635, KT215639-KT215643 (GONÇALVES et al., 2015)
*Rhipidomys sp.** (climbing mouse)	Free-range/NI	7/15 (46.7%)	*Mycoplasma* spp., (n) *M*. sp.	KT215630 (GONÇALVES et al., 2015)
*Rhipidomys macrurus** (Cerrado climbing mouse)	Free-range/NI	1/3 (33.3 %)	*Mycoplasma* spp., (n) *M*. sp.	KT215626 (GONÇALVES et al., 2015)
*Thrichomys sp.** (hairy mouse)	Free-range/NI	2/3 (66.6%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
*Thrichomys apereoides** (common punaré)	Free-range/NI	1/10 (10.0%)	*Mycoplasma* spp., (n) *M*. sp.	NI (GONÇALVES et al., 2015)
*Thrichomys fosteri** (Paraguayan punaré)	Free-range/NI	4/18 (22.2%)	*Mycoplasma* spp., (n) *M*. sp.	KT215634 (GONÇALVES et al., 2015)
*Thrichomys laurentius** (Pernambuco punaré)	Free-range/NI	2/17 (11.7%)	*Mycoplasma* spp., (n) *M*. sp.	KT215625 (GONÇALVES et al., 2015)
Order: Chiroptera				
Miniopterus schreibersii (Schreiber's bat)	Free-range/NI	22/30 (73.3%)	'*Ca*. M. hemohominis'	KM538692, KM538695-KM538697 (MILLÁN et al., 2015)
Miniopterus schreibersii (Schreiber's bat)	Free-range/NI	6/30 (20.0%)	'*Ca*. M. hemominiopterus'	KM538691, KM538698 (MILLÁN et al., 2015)
Myotis capaccinii (long-eared bat)	Free-range/NI	1/1 (100%)	'*Ca*. M. hemohominis'	KM538693 (MILLÁN et al., 2015)
Myotis lucifugus (little brown bat)	Free-range/NI	32/68 (47.0%)	(n) *M*. sp.	KF713538 (MASCARELLI et al., 2014)

* Wild mammal species of Brazil. Abbreviation: (n) = (novel); *M*.= *Mycoplasma*; '*Ca*. M.' = '*Candidatus* Mycoplasma'; NI = not informed.

Table 2. Hemoplasma species molecularly detected in wild mammals belonging to the order Carnivora.

Scientific name / Common name	**Origin / Status**	**Number / Prevalence**	**Hemoplasma species**	**Gen Bank / (Reference)**
Acinonyx jubatus (cheetah)	Free-range/NI	1/61 (1.6%)	*M. haemofelis/haemocanis* group	GU734681, GU734682 (KRENGEL et al., 2013)
Felis silvestris silvestris (wildcat)	Free-range/NI	1/31 (3.2%)	*M. haemofelis*	DQ825441, DQ859006 (WILLI et al., 2007a)
Felis silvestris silvestris (wildcat)	Free-range/NI	6/31 (19.3%)	'*Ca*. M. haemominutum'	DQ825442, DQ825443 (WILLI et al., 2007a)
Felis silvestris silvestris (wildcat)	Free-range/NI	11/31 (35.5%)	'*Ca*. M. turicensis'	DQ825449, DQ825450 (WILLI et al., 2007a)
*Leopardus pardalis** (ocelot)	Captive/NI	1/7 (14.3%)	*M. haemofelis*	NI (WILLI et al., 2007a)
*Leopardus pardalis** (ocelot)	Captive/NI	1/7 (14.3%)	'*Ca*. M. turicensis'	DQ825448 (WILLI et al., 2007a)
*Leopardus pardalis** (ocelot)	Captive/NI	4/7 (57.1%)	'*Ca*. M. haemominutum'	NI (WILLI et al., 2007a)
*Leopardus pardalis** (ocelot)	Captive/NI	10/43 (23.2%)	'*Ca*. M. haemominutum'	NI (ANDRÉ et al., 2011)
*Leopardus tigrinus** (oncilla)	Captive/NI	3/33 (9.1%)	'*Ca*. M. haemominutum'	DQ825439 (WILLI et al., 2007a)
*Leopardus tigrinus** (oncilla)	Captive/NI	3/39 (7.7%)	'*Ca*. M. haemominutum'	NI (ANDRÉ et al., 2011)
*Leopardus tigrinus** (oncilla)	Captive/NI	1/39 (2.6%)	*M. haemofelis*	NI (ANDRÉ et al., 2011)
*Leopardus wiedii** (margay)	Captive/NI	1/9 (11.1%)	*M. haemofelis*	DQ825438 (WILLI et al, 2007a)
*Leopardus wiedii** (margay)	Captive/NI	1/9 (11.1%)	'*Ca*. M. haemominutum'	DQ825440 (WILLI et al, 2007a)
Lynx lynx (Eurasian lynx)	Free-range/NI	4/36 (11.1%)	*M. haemofelis*	DQ825458, DQ859011 (WILLI et al., 2007a)

Scientific name / Common name	**Origin / Status**	**Number / Prevalence**	**Hemoplasma species**	**Gen Bank / (Reference)**
Lynx lynx (Eurasian lynx)	Free-range/NI	14/36 (38.9%)	'*Ca*. M. haemominutum'	DQ825456, DQ825457 (WILLI et al., 2007a)
Lynx lynx (Eurasian lynx)	Free-range/NI	2/36 (5.5%)	'*Ca*. M. turicensis'	NI (WILLI et al., 2007a)
Lynx pardinus (Iberian lynx)	Captive/NI	7/35 (20.0%)	*M. haemofelis*	DQ825447 (WILLI et al., 2007a)
Lynx pardinus (Iberian lynx)	Captive/NI	9/35 (25.7%)	'*Ca*. M. haemominutum'	DQ825444-DQ825446 (WILLI et al., 2007a)
Lynx pardinus (Iberian lynx)	Captive/NI	3/35 (8.6%)	'*Ca*. M. turicensis'	NI (WILLI et al., 2007a)
Lynx pardinus (Iberian lynx)	Free-range/NI	25/77 (32.5%)	*M. haemofelis*	NI (MELI et al., 2009)
Lynx pardinus (Iberian lynx)	Free-range/NI	27/77 (35.1%)	'*Ca*. M. haemominutum'	NI (MELI et al., 2009)
Lynx pardinus (Iberian lynx)	Free-range/NI	10/77 (13.0%)	'*Ca*. M. turicensis'	NI (MELI et al., 2009)
*Leopardus geoffroyi** (Geoffroy's cat)	Captive/NI	1/7 (14.3%)	'*Ca*. M. haemominutum'	NI (WILLI et al., 2007a)
Panthera leo (lion)	Free-range /NI	31/45 (68.9%)	*M. haemofelis*	DQ825451, DQ825453, DQ859007, DQ859009, DQ859010, DQ859012 (WILLI et al., 2007a)
Panthera leo (lion)	Free-range /NI	43/45 (95.5%)	'*Ca*. M. haemominutum'	DQ825452, DQ825455 (WILLI et al., 2007a)
Panthera leo (lion)	Free-range /NI	34/45 (75.5%)	'*Ca*. M. turicensis'	DQ825454 (WILLI et al., 2007a)
Panthera leo (lion)	Captive/NI	1/5 (20.0%)	'*Ca*. M. haemominutum'	NI (WILLI et al., 2007a)
Panthera leo (lion)	Captive/ Healthy	1/1 (100%)	'*Ca*. M. haemominutum'	NI (GUIMARÃES et al., 2007)
Panthera leo (lion)	Free-range/ Healthy and Sick	NI	'*Ca*. M. haemominutum'	NI (MUNSON et al., 2008)
*Panthera onca** (jaguar)	Captive/NI	4/14 (28.6%)	'*Ca*. M. haemominutum'	NI (ANDRÉ et al., 2011)

Scientific name / Common name	**Origin / Status**	**Number / Prevalence**	**Hemoplasma species**	**Gen Bank / (Reference)**
Panthera tigris (tiger)	Captive/Sick	2/28 (7.1%)	*M. haemofelis*	NI (HAEFNER et al., 2003)
*Puma concolor** (puma)	Captive/NI	1/2 (50.0%)	'*Ca*. M. haemominutum'	NI (WILLI et al., 2007a)
*Puma concolor** (puma)	Captive/NI	3/18 (16.7%)	'*Ca*. M. haemominutum'	NI (ANDRÉ et al., 2011)
*Puma yagouaroundi** (jaguarondi)	Captive/NI	2/25 (8.0%)	'*Ca*. M. haemominutum'	NI (ANDRÉ et al., 2011)
Prionailurus bengalensis euptilurus (leopard cat)	NI/NI	7/29 (24.1%)	*M. haemominutum*	EF198147, KP843885-KP843890 (HWANG et al., 2015)
Prionailurus bengalensis *euptilurus* (leopard cat)	NI/NI	1/29 (3.4%)	*M. haemofelis*	EF198144 (HWANG et al., 2015)
Prionailurus bengalensis euptilurus (leopard cat)	NI/NI	2/29 (6.9%)	*M. haemomuris*-like	KP843891, KP843892(HWANG et al., 2015)
Prionailurus iriomotensis (Iriomote cat)	Free-range/NI	3/31 (9.7%)	*M. haemofelis*	AB697740, AB697741 (HIRATA et al., 2012)
Prionailurus iriomotensis (Iriomote cat)	Free-range/NI	1/31 (3.2%)	'*Ca*. M. turicensis'	AB697739 (HIRATA et al., 2012)
Herpestes javanicus (Indian mongoose)	Free-range /Healthy	1/14 (7.1%)	'*Ca*. M. turicensis'	KJ530704 (SHARIFIYAZDI et al., 2014)
Meles meles anakuma (Japanese badger)	Free-range/NI	1/1 (100%)	'*Ca*. M. haemomeles'	AB848713 (HARASAWA et al., 2014)
*Canis lupus** (wolf)	Captive/NI	2/3 (66.7%)	*M*. sp.	NI (ANDRÉ et al., 2011)
Lycalopex fulvipes (Darwin's fox)	Free-range/NI	3/29 (10.3%)	(n) *M*. sp.	NI (CABELLO et al., 2013)
Lycalopex fulvipes (Darwin's fox)	Free-range/NI	5/29 (17.2%)	*M*. spp.	HF678195, HF679526 (CABELLO et al., 2013)
Lycalopex fulvipes (Darwin's fox)	Free-range/NI	8/29 (27.5%)	*M. haemocanis*	NI (CABELLO et al., 2013)
Lycalopex fulvipes (Darwin's fox)	Free-range/NI	1/29 (3.4%)	*M. haemofelis*	NI (CABELLO et al., 2013)
Nyctereutes procyonoides viverrinus (raccoon dog)	Free-range/NI	1/1 (100%)	*M. haemocanis*	AB848714 (HARASAWA et al., 2014)

Scientific name / Common name	**Origin / Status**	**Number / Prevalence**	**Hemoplasma species**	**Gen Bank / (Reference)**
*Speothos venaticus** (bush dog)	Captive/NI	2/27 (7.4%)	'*Ca*. M. haematoparvum'	NI (ANDRÉ et al., 2011)
Vulpes lagopus (Arctic fox)	Captive/NI	1/28 (3.6%)	*M. haemocanis*	NI (MASCARELLI et al., 2015)
Ursus thibetanus (black bear)	Free-range/NI	8/15 (53.3%)	(n) *M.* sp.	AB725596 (ISO et al., 2013)
Zalophus californianus (California sea lion)	Captive/ Healthy	17/137 (12.4%)	'Ca. M. haemozalophi sp. nov.'	GU124600–GU124614, GU904996-GU905012 (VOLOKHOV et al., 2011)

* Wild mammal species of Brazil. Abbreviation: (n) = (novel); *M.*= *Mycoplasma*; '*Ca*. M.' = '*Candidatus* Mycoplasma'; NI = not informed.

Table 3. Hemoplasma species molecularly detected in wild mammals belonging to the order Artiodactyla.

Scientific name / Common name	**Origin / Status**	**Number / Prevalence**	**Hemoplasma species**	**Gen Bank / (Reference)**
Order: Artiodactyla				
*Blastocerus dichotomus** (marsh deer)	Captive/NI	4/6 (66.7%)	*M. ovis*	HQ197743, HQ197746, HQ197747, HQ197750, HQ197751 (GRAZZIOTIN et al., 2011a)
*Blastocerus dichotomus** (marsh deer)	Captive/ Healthy and Sick	46/64 (71.9%)	*M. ovis*	HQ634378, HQ634379, HQ634381 (GRAZZIOTIN et al., 2011b)
*Blastocerus dichotomus** (marsh deer)	Captive/NI	1/64 (1.6%)	(n) *M*. sp.	HQ197747 (GRAZZIOTIN et al., 2011b)
*Blastocerus dichotomus** (marsh deer)	Captive/NI	5/64 (7.8%)	'*Ca*. M. erythrocervae'	NI (GRAZZIOTIN et al., 2011b)
Capricornis crispus (Japanese serow)	Free-range/NI	5/19 (26.3%)	*M. ovis*	AB571119 (OHTAKE et al., 2011)
Cervus Nippon (sika deer)	Free-range/ Healthy	2/147 (1.4%)	'*Ca*. M. erythrocervae',	AB558897, AB558898 (WATANABE et al., 2010)
Cervus Nippon (sika deer)	Free-range/ Healthy	1/147(0.7%)	'*Ca*. M. haemocervae'	AB558899 (WATANABE et al., 2010)
Cervus Nippon (sika deer)	Free-range/ Healthy	10/147(6.8%)	*Mycoplasma* sp.	NI (WATANABE et al., 2010)
Cervus nippon yesoensis (sika deer)	Captive/NI	12/51 (23.5%)	'*Ca*. M. haemocervae'	KF306246, KF306248-KF306250, KF306252, KF306253, AB836745-AB836747 (TAGAWA et al., 2014)
Cervus nippon yesoensis (sika deer)	Captive/NI	17/51 (33.3%)	'*Ca*. M. erythrocervae'	KF306247, KF306251, KF306254, AB836744, AB836748 (TAGAWA et al., 2014)
*Mazama americana** (red brocket deer)	Captive/NI	2/3 (66.7%)	*M. ovis*	HQ197748 (GRAZZIOTIN et al., 2011a)

Scientific name / Common name	**Origin / Status**	**Number / Prevalence**	**Hemoplasma species**	**Gen Bank / (Reference)**
*Mazama nana** (dwarf brocket deer)	Captive/NI	21/22 (95.4%)	*M. ovis*	HQ197742, HQ197744, HQ197745, HQ197749 (GRAZZIOTIN et al., 2011a)
Odocoileus virginianus (white-tailed deer)	Captive/ Sick	1/1 (100%)	*M. ovis*-like	FJ824847 (BOES et al., 2012)
Odocoileus virginianus (white-tailed deer)	Captive/ Healthy	7/8 (87.5%)	*M. ovis*-like	NI (BOES et al., 2012)
Odocoileus virginianus (white-tailed deer)	Free-range/ Healthy	65/73 (89.0%)	*M.* spp., (n) *M.* sp.	KC512402-KC512404, JQ61062, JQ610627, JQ610628 (MAGGI et al., 2013b)
*Ozotoceros bezoarticus** (pampas deer)	Free-range/NI	14/39 (35.9%)	*M. ovis*	HQ634377, HQ634380, HQ634382, HQ634383 (GRAZZIOTIN et al., 2011b)
Rangifer tarandus (reindeer)	NI/Sick	9/19 (47.4%)	'*Ca*. M. haemotarandirangiferis'	DQ524812-DQ524819 (STOFFREGEN et al., 2006)
Sus scrofa (wild boar)	Free-range and Captive/NI	36/359 (10.0%)	*M. suis*	FN391018-FN391020, FN436009-FN436019 (HOELZLE et al., 2010)
*Vicugna pacos (*alpaca)	NI/Sick	1/1 (100%)	'*Ca*. M. haemolamae'	AF306346 (MESSICK, et al., 2002)
*Vicugna pacos (*alpaca)	Captive/NI	NI	'*Ca*. M. haemolamae'	NI (KAUFMANN et al., 2010)
*Vicugna pacos (*alpaca)	NI/Healthy	35/131(26.7 %)	'*Ca*. M. haemolamae'	NI (CROSSE et al., 2012)
*Vicugna pacos (*alpaca)	NI/Sick	1/1 (100%)	'*Ca*. M. haemolamae'	JF495171 (CROSSE et al., 2012)
*Vicugna pacos (*alpaca)	Captive/Healthy and Sick	11/24 (45.8%)	*'Ca*. M. haemolamae'	GU047355-GU047356 (MELI et al., 2010)
*Vicugna pacos (a*lpaca)	Captive/Sick	1/1 (100%)	*M. haemolamae*	NI (ALMY et al., 2006)
*Vicugna pacos (*alpaca)	Captive/ Healthy	10/108 (9.3%)	*M. haemolamae*	NI (TORNQUISt et al., 2010)
Lama glama (llama)	Captive/ Healthy	12/76 (15.8%)	*M. haemolamae*	NI (TORNQUIST et al., 2010)
Lama glama (llama)	Captive/NI	NI	'*Ca*. M. haemolamae'	NI (KAUFMANN et al., 2010)

* Wild mammal species of Brazil. Abbreviation: (n) = (novel); *M.*=*Mycoplasma*; '*Ca*. M'.= '*Candidatus* Mycoplasma'; NI = not informed.

Table 4. Hemoplasma species detected in different wild mammals around the world.

Hemoplasma species	**Host wild animal species**
Mycoplasma spp.	black tamarin (*Saguinus niger*), monkey (*Sapajus* sp.), capuchin monkey (*Sapajus apella*), sharp-toothed akodont (*Akodon* sp.), montane grass mouse (*Akodon montensis*), vesper mouse (*Calomys sp.*), Cerqueira's vesper mouse (*Calomys cerqueirae*), delicate vesper mouse (*Calomys tener*), striped Atlantic forest rat (*Delomys dorsalis*), capybara (*Hydrochaeris hydrochaeris*), wood mouse (*Hylaeamys sp.*), house mouse (*Mus musculus*), hairy-tailed bolo mouse (*Necromys lasiurus*), water rat (*Nectomys sp.*), small-footed bristly mouse (*Nectomys rattus*), scaly-footed water rat (*Nectomys squamipes*), arboreal rice rat (*Oecomys sp.*), pigmy rice rat (*Oligoryzomys sp.*), yellow pygmy rice rat (*Oligoryzomys flavescens*), Fornes's colilargo (*Oligoryzomys fornesi*), black-footed pygmy rice rat (*Oligoryzomys nigripes*), hocicudo (*Oxymycterus sp.*), brown sewer rat (*Rattus norvegicus*), black rat (*Rattus rattus*), climbing mouse (*Rhipidomys sp.*), Cerrado climbing mouse (*Rhipidomys macrurus*), hairy mouse (*Thrichomys sp.*), common punaré (*Thrichomys apereoides*), Paraguayan punaré (*Thrichomys fosteri*), Pernambuco punaré (*Thrichomys laurentius*), little brown bat (*Myotis lucifugus*), Darwin's fox (*Lycalopex fulvipes*), black bear (*Ursus thibetanus*), white-tailed deer (*Odocoileus virginianus*).
(n) *Mycoplasma* sp.	black howler monkey (*Alouatta caraya*), black tamarin (*Saguinus niger*), monkey (*Sapajus* sp.), capuchin monkey (*Sapajus apella*), sharp toothed akodont (*Akodon* sp.), montane grass mouse (*Akodon montensis*), vesper mouse (*Calomys sp.*), Cerqueira's vesper mouse (*Calomys cerqueirae*), delicate vesper mouse (*Calomys tener*), striped Atlantic forest rat (*Delomys dorsalis*), capybara (*Hydrochaeris hydrochaeris*), wood mouse (*Hylaeamys sp.*), house mouse (*Mus musculus*), hairy-tailed bolo mouse (*Necromys lasiurus*), water rat (*Nectomys sp.*), small-footed bristly mouse (*Nectomys rattus*), scaly-footed water rat (*Nectomys squamipes*), arboreal rice rat (*Oecomys sp.*), pigmy rice rat (*Oligoryzomys sp.*), yellow pygmy rice rat (*Oligoryzomys flavescens*), Fornes's colilargo (*Oligoryzomys fornesi*), black-footed pygmy rice rat (*Oligoryzomys nigripes*), hocicudo (*Oxymycterus sp.*), brown sewer rat (*Rattus norvegicus*), black rat (*Rattus rattus*), climbing mouse (*Rhipidomys sp.*), Cerrado climbing mouse (*Rhipidomys macrurus*), hairy mouse (*Thrichomys sp.*), common punaré (*Thrichomys apereoides*), Paraguayan punaré (*Thrichomys fosteri*), Pernambuco punaré (*Thrichomys laurentius*), little brown bat (*Myotis lucifugus*), Darwin's fox (*Lycalopex fulvipes*), black bear (*Ursus thibetanus*), white-tailed deer (*Odocoileus virginianus*), pampas deer (*Ozotoceros bezoarticus*).
Mycoplasma coccoides	field mouse (*Apodemus* sp.), capybara (*Hydrochaeris hydrochaeris*).

Hemoplasma species	**Host wild animal species**
Mycoplasma haemofelis	European wildcat (*Felis silvestris silvestris*), ocelot (*Leopardus pardalis*), oncilla (*Leopardus tigrinus*), margay (*Leopardus wiedii*), Eurasian lynx (*Lynx lynx*), Iberian lynx (*Lynx pardinus*), lion (*Panthera leo*), tiger (*Panthera tigris*), Iriomote cat (*Prionailurus iriomotensis*), Darwin's fox (*Lycalopex fulvipes*).
Mycoplasma haemofelis/haemocanis group	Namibian cheetah (*Acynonyx jubatus*), reindeer (*Rangifer tarandus*).
Mycoplasma haemocanis	Darwin's fox (*Lycalopex fulvipes*), raccoon dog (*Nyctereutes procyonoides*), Arctic fox (*Vulpes lagopus*).
Mycoplasma haemolamae	alpaca (*Vicugna pacos*), llama (*Lama glama*).
Mycoplasma haemominutum	leopard cat (*Prionailurus bengalensis euptilurus*).
Mycoplasma haemomuris	brown sewer rat (*Rattus norvegicus*).
Mycoplasma haemomuris-like	leopard cat (*Prionailurus bengalensis euptilurus*).
Mycoplasma ovis	marsh deer (*Blastocerus dichotomus*), Japanese serow (*Capricornis crispus*), red brocket deer (*Mazama americana*), dwarf brocket deer (*Mazama nana*), pampas deer (*Ozotoceros bezoarticus*), reindeer (*Rangifer tarandus*).
Mycoplasma ovis-like	white-tailed deer (*Odocoileus virginianus*).
Mycoplasma suis	wild boar (*Sus scrofa*).
Mycoplasma wenyonii	reindeer (*Rangifer tarandus*).
'*Candidatus* Mycoplasma aoti'	owl monkey (*Aotus trivirgatus*).
'*Candidatus* Mycoplasma erythrocervae'	marsh deer (*Blastocerus dichotomus*), sika deer (*Cervus nippon*), sika deer (*Cervus nippon yesoensis*).
'*Candidatus* Mycoplasma haemocervae'	sika deer (*Cervus nippon*), sika deer (*Cervus nippon yesoensis*).
'*Candidatus* Mycoplasma haemodidelphidis'	opossum (*Didelphis virginiana*).
'*Candidatus* Mycoplasma haemolamae'	alpaca (*Vicugna pacos*), llama (*Lama glama*).

Hemoplasma species	**Host wild animal species**
'*Candidatus* Mycoplasma haemomacaque'	cynomolgus monkey (*Macaca fascicularis*), Japanese monkey (*Macaca fuscata*).
'*Candidatus* Mycoplasma haemominutum'	European wildcat (*Felis silvestris silvestris*), ocelot (*Leopardus pardalis*), oncilla (*Leopardus tigrinus*), margay (*Leopardus wiedii*), Eurasian lynx (*Lynx lynx*), Iberian lynx (*Lynx pardinus*), Geoffroy's cats (*Leopardus geoffroyi*), lion (*Panthera leo*), jaguar (*Panthera onca*), puma (*Puma concolor*), jaguarondi (Puma *yagouaroundi*), European wolf (*Canis lupus*).
'*Candidatus* Mycoplasma haemomeles'	Japanese badger (*Meles meles anakuma*).
'*Candidatus* Mycoplasma haemoparvum'	bush dog (*Speothos venaticus*).
'*Candidatus* Mycoplasma haemotarandirangiferis'	reindeer (*Rangifer tarandus*).
'*Candidatus* Mycoplasma haemozalophi sp. nov'.	California sea lion (*Zalophus californianus*).
'*Candidatus* Mycoplasma hemohominis'	Schreiber's bat (*Miniopterus schreibersii*), long-eared bat (*Myotis capaccinii*).
'*Candidatus* Mycoplasma hemominiopterus'	Schreiber's bat (*Miniopterus schreibersii*).
'*Candidatus* Mycoplasma kahanei'	squirrel monkey (*Saimiri sciureus*), black howler monkey (*Alouatta caraya*).
'*Candidatus* Mycoplasma turiscensis'	European wildcat (*Felis silvestris silvestris*), ocelot (*Leopardus pardalis*), Eurasian lynx (*Lynx lynx*), Iberian lynx (*Lynx pardinus*), lion (*Panthera leo*), Iriomote cat (*Prionailurus iriomotensis*).
'*Candidatus* Mycoplasma turicensis'-like	Indian mongoose (*Herpestes javanicus*).

Table 5. Meta-analysis results from all animals, wild and captive in general and according to the orders of Mammalia in which they were classified.

	All animals			Free range			Captive		
	Prevalence (%)	**95% CI**	**I^2***	**Prevalence (%)**	**95% CI**	**I^2***	**Prevalence (%)**	**95% CI**	**I^2***
All animals	28.92	24.53 - 33.74	p<0.001	31.00	24.97 – 37.76	p<0.001	22.33	17.20 – 28.47	p<0.001
Order Artiodactyla	32.65	25.98 - 40.10	p=0.091	21.22	9.63 – 40.50	p<0.001	37.54	25.29 – 51.62	p=0.108
Order Carnivora	13.26	10.58 - 16.48	p=0.016	12.53	8.31 – 18.46	p=0.081	14.82	10.99 – 19.68	p=0.051
Order Chiroptera	46.30	21.63 - 72.93	p=0.001	46.30	21.62 – 72.93	p=0.001	-	-	-
Order Primates	42.37	21.39 - 66.51	p=0.056	-	-	-	42.37	21.39 – 66.51	p=0.056
Order Rodentia	33.16	27.33 - 39.56	p=0.089	32.34	26.15 – 39.23	p=0.067	40.29	24.39 – 58.54	p=0.432

*I-square: test of heterogeneity

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3 HEMOPLASMAS (HEMOTROPIC MYCOPLASMA) IN BATS OF SOUTHERN BRAZIL

3.1 ABSTRACT

Hemotropic mycoplasmas (hemoplasmas) are worldwide distributed bacteria affecting domestic and wildlife animals besides human beings. Although already described in non-hematophagous bats, transmission risk and zoonotic potential remain to be fully established. Accordingly, 10 blood samples were taken from 6 males common vampire bat (*Desmodus rotundus*), 2 males hairy-legged vampire bat (*Diphylla ecaudata*) and 2 females Pallas's non-hematophagous mastiff bat (*Molossus* sp.) at Curitiba's region, Parana State, southern Brazil. All samples were tested for a specific PCR protocol to hemoplasma amplification. In overall, 8/10 (80.0%) bats tested positive to *Mycoplasma* sp. including 5/6 (83.3%) *Desmodus rotundus*, 2/2 (100%) *Diphylla ecaudata* and 1/2 (50.0%) *Molossus* sp. The analyses of the partial sequence of 16S rRNA gene have identified a potentially novel hemoplasma species infecting bats at Curitiba's region, Parana State, southern Brazil.

Keywords: hemotropic mycoplasmas, *Desmodus rotundus*, *Diphylla ecaudata*, *Molossus* sp.

3.2 INTRODUCTION

Hemotropic mycoplasmas (hemoplasmas) are epicellular erythrocytic bacteria and the infection can range from asymptomatic to illnesses characterized by hemolytic anemia [1, 2]. '*Candidatus* Mycoplasma hemohominis' has been described in Schreiber's bat (*Miniopterus schreibersii*) and in long-eared bat (*Myotis capaccinii*) in Spain [3], '*Candidatus* Micoplasma hemominiopterus' in Schreiber's bat (*Miniopterus schreibersii*) [3], and a novel hemotropic Mycoplasma species in little brown bats *Myotis lucifugus* in the United States of America [2].

Bats are among the most eco-epidemiologically important mammals, owing to their presence in human settlements and animal keeping facilities. Roosting of bats in buildings may bring pathogens of public health concern into the domestic animals environment and urban areas [4]. Bats live in large or small colonies, could be found in caves, fissures, forest, tree hollows, old wells, mineshafts, and abandoned buildings [5].

A study regarding the existence and distribution of the bat species occurring in the State of Paraná, Brazil, was performed using the collection belonging to the Capao da Imbuia Natural History Museum (Museu de História Natural Capão da Imbuia, MHNCI). In Curitiba, fifty-three species in five families were identified, the Phyllostomidae present the highest

abundance of species 25 (47%) followed by the Molossidae 13 (24%), Vespertilionidae 12 (23%), Noctilionidae 2 (4%), and Emballonuridae 1 (2%) [6].

The objective of this study was to determine the molecular frequency of hemotropic mycoplasma species in 10 free-ranging bats at Curitiba's region, Parana State, Southern Brazil.

3.3 MATERIALS AND METHODS

The present study has been approved by the Bioscience Institute/UNESP Ethics Committee on Use of Animals (CEUA) (protocol number 809) and Chico Mendes Institute of Biodiversity Conservation (ICMBio), System of Authorization and Information in Biodiversity (SISBIO) under the protocol 51714-1.

3.3.1 Sampling

Blood samples (n=10) were taken from eight hematophagous bats: six males common vampire bat (*Desmodus rotundus*; Family Phyllostomidae), two males hairy-legged vampire bat (*Diphylla ecaudata*; Family Phyllostomidae); and two no-hematophagous females Pallas's mastiff bat (*Molossus* sp.; Family Molossidae), at Curitiba's region, Parana State, southern Brazil.

For anesthesia, bat cages were put inside a plastic container and isoflurane was infused with a machine with oxygen. Sedation maintenance was performed using inhalation mask. Intracardiac puncture was performed to obtain blood, sequentially, the bats were euthanized with lethal dose of intracardiac potassium chloride. Blood samples were stored at -20ºC until processed.

3.3.2 Microscopic detection

Blood smears of two *Desmodus rotundus*, prepared immediately after blood collection, were stained with May-Grünwald-Giemsa using an automated slide stainer (Sysmex XE-2100, Sysmex Corporation, Japan). The smears were examined using light microscopy at 1,000x magnification for the presence of hemoplasma (Confocal and Conventional Fluorescence Microscopy Multi-user Laboratory, Department of Biological Sciences, Federal University of Paraná State, Brazil). Images were edited for publication using GIMP v.2.8.16 software (available at http://www.gimp.org/).

3.3.3 PCR assays

DNA was extracted from 200 µL blood using a commercially available kit according to the manufacturer's instructions (Illustra Blood Genomic Prep Mini Spin Kit, GE Healthcare, Chalfont, St. Giles, UK). Negative control purifications using ultrapure water were performed in parallel to monitor cross contamination.

A PCR for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase GAPDH), was performed to ensure successful DNA extraction, as previously described [7]. Thereafter, samples were screened by conventional pan-hemoplasma PCR targeting the 16S rDNA regions specific for hemoplasmas, using primers previously described [8]. '*Candidatus* M. haemovis'-positive goat blood sample and nuclease-free water were used as positive and negative control, respectively.

3.3.4 16S rRNA gene sequencing

By using universal primers for the 16S rRNA (Reference), samples from two bats of the species *Desmodus rotundus* that tested positive for *Mycoplasma* sp in the first PCR reaction, were amplified using Platinum® Taq High Fidelity DNA Polymerase. PCR products of 745 bp were purified from the 1.5% agarose gel (PureLink® Quick Gel Extraction Kit, Invitrogen DNA, Carlsbad, CA, USA) and sequenced in both sense and antisense directions (Laboratório de Virologia, Universidade Estadual de Londrina – UEL, Londrina, PR, Brazil).

3.3.5 Phylogenetic analysis

Sense and antisense sequences were manually examined and edited using MEGA 6.06 [9] and the consensus sequence was compared on BLASTN [10] to verify identity percentage with other sequences deposited in the GenBank nucleotide database. The hemoplasma 16S rRNA gene sequence was aligned with known sequences obtained from GenBank (Mafft v7.300b) [11] and then manually improved (MEGA 6) [9]. The best nucleotide substitution model was determined (MEGA 6) [9] and was set as GTR+G+I in the maximum likelyhood (ML) phylogenetic estimation (RAxML v. 8.2.8) [12], CIPRES Science Gateway [13], including 1000 bootstrap replicates. Reconstruction was visualized with Fig. Tree 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree) and the final layout was done with Inkscape 0.91 (www.inkscape.org).

3.3.6 Nucleotide sequence accession numbers

The nucleotide sequences of the hemoplasmas isolates from bats were submitted to the GenBank database under the accession number KX722541.

3.4 RESULTS AND DISCUSSION

Hemoplasmas were present in the blood smears prepared from the two *Desmodus rotundus*. Both samples were positive for hemoplasma in the PCR assay. The structures were compatible with other hemoplasmas reports [1]. They were spherical or rod-shaped, individually or in chains on the surface of erythrocytes (Fig.1).

The frequency of positive bats to *Mycoplasma* sp. was 83,3% (5/6) in *Desmodus rotundus*, 100% (2/2) for *Diphylla ecaudata*, and 50.0% (1/2) for *Molossus* sp. by conventional PCR assay. In *Miniopterus schreibersii* has shown 22/30 (73.3%) and in *Myotis capaccinii* 1/1(100%) animals with '*Candidatus* Mycoplasma hemohominis', and in *Miniopterus schreibersii* 6/30 (20.0%) to '*Candidatus* Micoplasma hemominiopterus' [3]. In *Myotis lucifugus* has shown 32/68 (47.0%) animals with a novel hemotropic Mycoplasma species [2].

One 16S rRNA mycoplasma gene from a *Desmodus rotundus* sample was successfully sequenced. The BLAST analysis of the obtained sequence (745 bp) (GenBank accession number KX722541) showed the highest identity to *Mycoplasma* sp. detected in rodents from Brazil and Hungary (95%, GenBank no. KT215636, KM203857, KJ739311, KC863983) and detected in a coati from Brazil (95%, GenBank no. KU554425).

The ML analysis comprised 43 taxons of 805 characters. The best scoring phylogenetic tree (Fig 2), with ML bootstrap values equal or greater than 50% given near each node, showed two characteristic hemoplasma clusters, named *M. suis* and *M. haemofelis* [14, 15]. Bat hemoplasma sequence clustered in the *M. haemofelis* cluster as basal clade to hemoplasmas detected in a coati and capybaras from Brazil (GenBank no. KU554425, FJ667773 and FJ667774), in a sister clade to hemoplasmas detected in rodents from Brazil and Hungary (GenBank no. KT215636, KJ739311, KM203857, KC863983). Other hemoplasmas detected in bats were positioned in a separated clade within the *M. haemofelis* group, which also comprise hemoplasmas detected in primates.

The analyses of the partial sequence of 16S rRNA gene have identified a potentially novel hemoplasma species infecting bat. It was most closely related to hemotropic *Mycoplasma* sp. found in rodents from different geographical regions [16, 17, 18, 19].

Similarities less than 97% in the 16S rRNA gene sequences between two bacterial isolates have suggested that they may belong to different species [20].

3.5 CONCLUSION

The analyses of the partial sequence of 16S rRNA gene have identified a potentially novel hemoplasma species infecting common vampire bat (*Desmodus rotundus*), hairy-legged vampire bat (*Diphylla ecaudata*) and Pallas's mastiff bat (*Molossus* sp.), at Curitiba's region, Parana State, southern Brazil.

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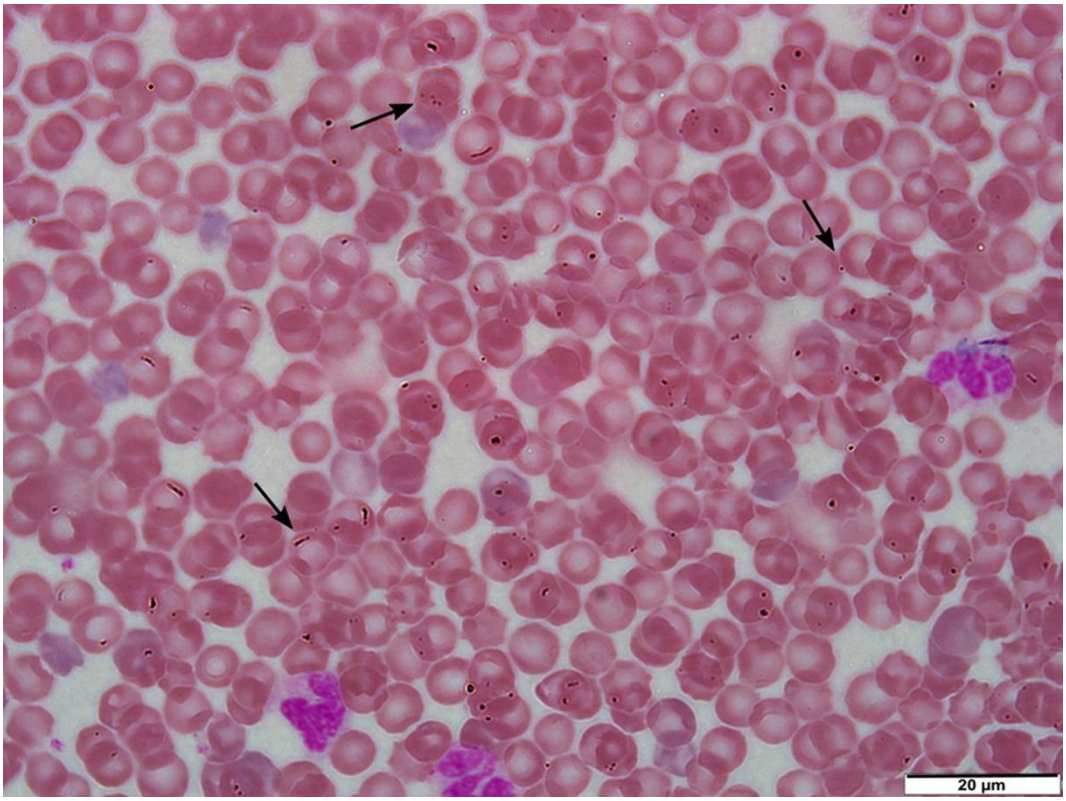

Figure 1. Light microscopy image of May-Grünwald-Giemsa-stained blood smears from *Desmodus rotundus* (1,000x). Hemoplasmas are attached to erythrocytes (arrows). Bar = 20 µm.

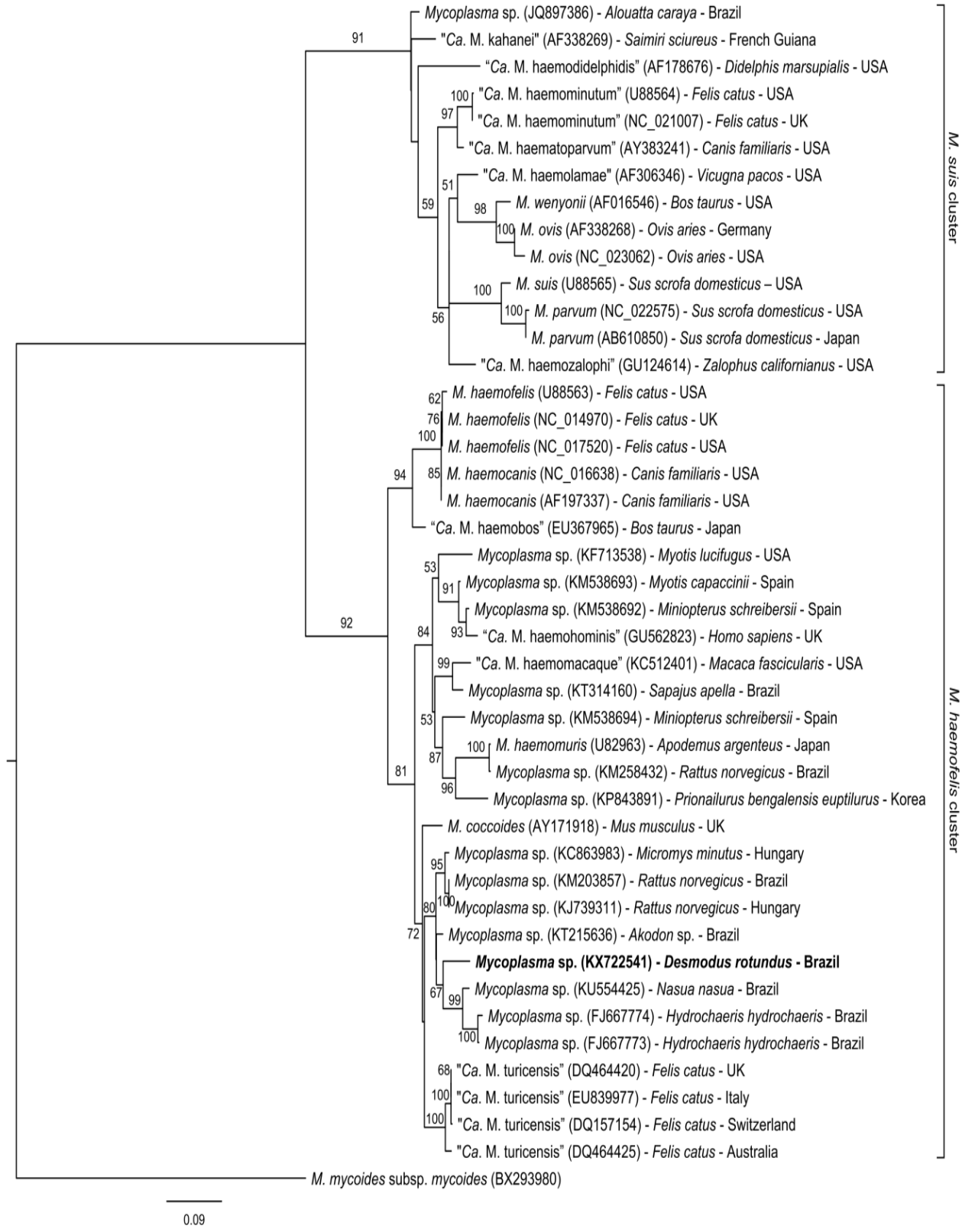

Figure 2. Phylogenetic relationships among 42 hemoplasmas inferred from 16S rRNA gene sequences. Maximum likelihood tree was constructed from 805 aligned characters. Data set was resampled 1000 times and bootstrap support values ≥ 50% are indicated at the nodes. Bat hemoplasma is in bold. Species names are followed by GenBank accession number, host and country of origin. The tree was rooted to *Mycoplasma mycoides* subsp. *mycoides* (GenBank BX293980). The scale bar represents the expected number of changes per site.

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4 MOLECULAR SCREENING FOR HEMOTROPIC MYCOPLASMAS IN CAPTIVE BARBARY SHEEP (*Ammotragus lervia*) IN SOUTHERN BRAZIL

4.1 ABSTRACT

Hemotropic mycoplasmas (hemoplasmas) have been described as a potential cause of hemolytic anemia in domestic and wild mammal species. Although African Barbary sheep (*Ammotragus lervia*) are red-listed as vulnerable by the International Union for Conservation of Nature (IUCN), no hemoplasma survey has been conducted to date on this species. Accordingly, the aim of this study was to perform a PCR protocol for *Mycoplasma ovis* on 12 captive Barbary sheep at Curitiba Zoo, in southern Brazil. Blood samples were collected, DNA extracted and a PCR protocol for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was performed on all samples to ensure amplifiable DNA. Subsequently, all the samples were tested and found to be negative using a specific PCR protocol for *M. ovis* detection and amplification. Notwithstanding the negative results, molecular pathogen surveys on Barbary sheep and other exotic wild mammals may provide insights regarding infection of endangered species caused by captivity stress in association with exposure to new pathogens worldwide.

Keywords: Hemoplasmas, eperythrozoonosis, aoudads, hemolytic anemia.

4.2 DEVELOPMENT

Hemotropic mycoplasmas (hemoplasmas) have been described as pleomorphic and uncultivable bacteria that adhere to the surface of red blood cells in several domestic and wild mammals. They may cause hemolytic anemia and/or subclinical infections (MESSICK, 2004). *Mycoplasma ovis* (formerly *Eperythrozoon ovis*) is the main species infecting small ruminants.

However, this organism has also been described in other members of the family Bovidae such as the Japanese serow (*Capricornis crispus*) (OHTAKE et al., 2011), marsh deer (*Blastocerus dichotomus*), red brocket deer (*Mazama americana*), dwarf brocket deer (*Mazama nana*), pampas deer (*Ozotoceros bezoarticus*) (GRAZZIOTIN et al., 2011a; GRAZZIOTIN et al., 2011b) and reindeer (*Rangifer tarandus*) (STOFFREGEN et al., 2006).

Infection by other hemoplasmas have also been described in wild members of the family Bovidae, including *Mycoplasma ovis*-like in white-tailed deer (*Odocoileus*

virginianus) (BOES et al., 2012), '*Candidatus* Mycoplasma erythrocervae' in marsh deer (GRAZZIOTIN et al., 2011b) and sika deer (*Cervus nippon*) (WATANABE et al., 2010; TAGAWA et al., 2014), '*Candidatus* Mycoplasma haemocervae' in sika deer (WATANABE et al., 2010, TAGAWA et al., 2014) and *Mycoplasma* sp. in pampas deer (GRAZZIOTIN et al., 2011b) and white-tailed deer (MAGGI et al., 2013).

Barbary sheep (*Ammotragus lervia*) or aoudads belong to the subfamily Caprinae, sharing a series of characteristics in common with domestic mammals of the genera *Capra* and *Ovis* (NGUYEN et al., 1980; CASSINELLO, 1998, GONZÁLEZ-CANDELA et al., 2004). Despite being red-listed as vulnerable by the International Union for Conservation of Nature (IUCN, 2016), no hemoplasma survey has been conducted to date on this animal species.

Accordingly, the aim of this study was to screen captive Barbary sheep at Curitiba Zoo, in southern Brazil, for *Mycoplasma* sp. infection using a previously described pan-hemoplasma PCR assay (DIECKMANN et al., 2010; VIEIRA et al., 2015b). A total of 12 EDTA-blood samples previously surveyed for other pathogens (MORIKAWA et al., 2014) were used in this study. All the samples were stored at -80 °C until molecular procedures were performed.

DNA was extracted from 200 µL of blood using a commercially available kit (Illustra Blood Genomic Prep Mini Spin Kit, GE Healthcare, Chalfont St. Giles, UK), in accordance with the manufacturer's instructions. Negative control purifications using ultrapure water were performed in parallel, in order to monitor cross-contamination. PCR for the housekeeping gene of all mammal species, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed to ensure successful DNA extraction, as previously described (BIRKENHEUER et al., 2003). Thereafter, samples were screened by means of conventional pan-hemoplasma PCR targeting the 16S rDNA regions specific for hemoplasmas, using primers that had previously been described (DIECKMANN et al., 2010). A *M. ovis*-positive goat blood sample and nuclease-free water were used as positive and negative controls, respectively. Another study on wild Japanese serows showed that 5/19 animals (26.3%) were infected with *M. ovis* (OHTAKE et al., 2011).

Although housekeeping gene DNA was successfully amplified, all the Barbary sheep samples tested negative for *Mycoplasma* sp. In the present study, the animals were clinically healthy and not infested by ectoparasites, which may explain the negative results. However, we cannot rule out the possibility that these Barbary sheep may have been infected with an as

yet undescribed hemoplasma species that might not have been amplified by the primer set that was applied.

Among the potential causes of negative results from tests for hemoplasmas, previous reports have suggested that these include healthy animals (VIEIRA et al., 2015a, VIEIRA et al., 2015b); low parasitemia (VIEIRA et al., 2011); sample size and type of population (VIEIRA et al., 2015a); climatic conditions, presence of the vector and hemoplasma sequestration in tissues (NOVACCO et al., 2013); testing conducted during and immediately after antibiotic treatment (BERENT et al., 1998); and use of inappropriate laboratory tests (VIEIRA et al., 2011).

Notwithstanding the negative results, molecular pathogen surveys on Barbary sheep and other exotic wild mammals may provide insights regarding infection of endangered species caused by captivity stress in association with exposure to new pathogens worldwide. Further analysis should be conducted to elucidate whether Barbary sheep could be infected by an as yet undescribed hemoplasma species that cannot be amplified through the molecular assays that were applied here.

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5 GENERAL CONCLUSIONS

Viral, bacterial and parasitic diseases that occur in wild mammals, in free-range or captive, can be present zoonotic character, such as rabies, leptospirosis, leishmaniasis and toxoplasmosis.

In this thesis, in the review about by molecular methods the presence of hemotropic mycoplasmas in native and exotic wild mammals the prevalence of 29.92% for all reported animals; 31.00% for wild animals and 22.33% for captive animals.

The hemotropic mycoplasma was investigate in bats (*Desmodus rotundus, Diphylla ecaudata* and *Molossus* sp.) at Curitiba's region, and in Barbary sheep (*Ammotragus lervia*) at Curitiba Zoo, in southern Brazil. In overall, 8/10 (80.0%) bats tested positive to *Mycoplasma* sp. including 5/6 (83.3%) *Desmodus rotundus*, 2/2 (100%) *Diphylla ecaudata* and 1/2 (50.0%) *Molossus* sp. The analyses of the partial sequence of 16S rRNA gene have identified a potentially novel hemoplasma species infecting bats at Curitiba's region, Parana State, southern Brazil.

The PCR protocol for *Mycoplasma ovis* was investigate on 12 captive Barbary sheep at Curitiba Zoo, in southern Brazil. All the Barbary sheep samples tested negative for *Mycoplasma* sp. In the present study, the animals were clinically healthy and not infested by ectoparasites, which may explain the negative results. However, we cannot rule out the possibility that these Barbary sheep may have been infected with an as yet undescribed hemoplasma species that might not have been amplified by the primer set that was applied.

Since certain hemoplasmas may be considered to have clinical relevance and given the wide range of wild mammal hosts harboring this relatively simple form of life, hemoplasmas show great skill as opportunistic infection agents, along with unique cross-barrier ability for different host species.

Continuing research on hemoplasmas in wild mammals may provide comparative insights that fill several pathophysiological gaps such as in relation to their transmission and role in wildlife, livestock and companion animals, as well as in humans. Further studies should be conducted through a multidisciplinary approach, in order to fully establish reliable and affordable methods for diagnosis, treatment, monitoring and prevention of hemoplasma species infections.

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7 SUPPLEMENT

SUPPLEMENT 1

7.1 PATHOGENS IN AOUDADS (*Ammotragus lervia*): A REVIEW

ABSTRACT

Studies have been conducted in order to establish the role of wildlife as a reservoir of pathogens for humans and domestic animals. The aim of this study was to review the pathogens in aoudads (*Ammotragus lervia*). This is a review of articles published between September 1959 and October 2016, which were identified through a computerized search in the PubMed and SciELO electronic databases. The descriptor search in Medline and SciELO resulted in a total of 182 articles and 21 articles reporting pathogens. Pathogens were presented according to the origin of the animals (free-ranging or captive), country, health status and methods of detection. It was verified that some pathogens detected in aoudads, such as *Mycobacterium tuberculosis* and *Toxoplasma gondii*, may also infected domestic animals and human being. Then these wild animals could have an excellent reservoir potential for some pathogens spread.

Keywords: Pathogens, Hematology, Aoudad, Barbary sheep, *Ammotragus lervia*.

INTRODUCTION

Aoudad (Barbary sheep, uaddan, arui) native of North Africa, belong to the Class Mammalia, Order Artiodactyla, Family Bovidae, Subfamily Caprinae, Genus *Ammotragus*, Species *A.lervia*, Palla, 1777 (GRAY; SIMPSON, 1980; CASSINELLO, 1998; WILSON & REEDER, 1993). *Ammotragus lervia* have a series of characterístics in common with animals of species in the genuses *Capra* and *Ovis* (NGUYEN et al., 1980; CASSINELLO, 1998, GONZÁLEZ-CANDELA et al., 2004). In Barbary sheep the amino acid sequencing has indicated several differences in the HbB-chains from that of sheep and goats (CASSINELLO, 1998).

This species is classified as "vulnerable" (VU A2cd) by the IUCN (International Union for Conservation of Nature) in Africa its native range, but in Europe it is considered a biological invader (IUCN, 2006).

Aoudad (*Ammotragus lervia*) are an exotic species, native to North Africa, and are one of the most common exotic ungulates kept in zoos worldwide (YERUHAM et al., 2004).

Wildlife constitutes a large reservoir, often unknown, of emerging zoonotic infectious diseases (CHOMEL et al., 2007).

METHODOLOGY

The present descriptive study was conducted through a review of articles published between September 1959 and October 2016, which were identified through a computerized search in the PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and SciELO (http://www.scielo.org/) electronic databases, using the following general descriptors (keywords): Barbary sheep, *Ammotragus lervia*, Aoudad, "Parasites *Ammotragus lervia*". The same descriptors in English were also searched for in the SciELO database, with additional Portuguese and Spanish translations as keywords.

RESULTS

The descriptor search in Medline and SciELO resulted in a total of 182 articles and pathogens in *Ammotragus lervia* resulting in a total of 21 articles.

No reports were found using "Babesia in *Ammotragus lervia"*, "Babesia in aoudads", "Babesia in Babary sheep", "Trypanosoma in *Ammotragus lervia"*, "Trypanosoma in aoudads", "Trypanosoma in Barbary sheep", "Plasmodium in *Ammotragus lervia*", "Plasmodium in aoudads", "Plasmodium in Babary sheep".

Hematologic values in adults captive aoudad (*Ammotragus lervia*) is presented (Table 1) with the purpose to assist at the monitoring of these animals.

Virus, bacteria, protozoa, helminth and ectoparasites in aoudad (*Ammotragus lervia*) are shown in captive (Table 2) and free range (Table3).

It was verified that the following pathogens tested negative for *Ammotragus lervia*: virus: bovine virus diarrhea – BVD antibodies, vesicular stomatitis (VS) antibodies (HAMPY et al., 1979); bactéria: *Anaplasma* sp. antibodies, *Leptospira pomona, L. icterohemorrhagiae, L. canicola, L.hardjo, L. grippotyphosa* sorobodies, *Brucella* sp. sorobodies (HAMPY et al., 1979); *Brucella melitensis* antibodies, *Chlamydophila abortus* antibodies, bovine viral diarrhoea/border disease viruses BVDV-BDV antibodies (CANDELA et al., 2009); *Mycobacterium tuberculosis* (PORTAS et al, 2009); *Brucella* sp. antibodies (MUÑOZ et al, 2010); *Mycobacterium avium* ssp. *paratuberculosis* (MAP) (MÜNSTER et al., 2013); fungi: *Coccidioides* sp. antibodies (HAMPY et al., 1979); protozoa: *Neospora caninum* antibodies (SEDLÁK et al, 2006). In a recent study with aoudads in Brazil, hemotropic mycoplasma tested negative (SANTOS et al., unpublished data).

DISCUSSION AND CONCLUSION

The following questions should be considered about pathogens in wildlife:

Emerging infectious diseases (EIDs) of free-living wild animals can be classified into three major groups on the basis of key epizootiological criteria: EIDs associated with "spill-over" from domestic animals to wildlife populations living in proximity; EIDs related directly to human intervention, via host or parasite translocations; and EIDs with no overt human or domestic animal involvement. These phenomena have two major biological implications: first, many wildlife species are reservoirs of pathogens that threaten domestic animal and human health; second, wildlife EIDs pose a substantial threat to the conservation of global biodiversity (DASZAK et al., 2000).

Many wild reservoir hosts are increasing in number and geographic range, thus increasing intra- and interspecies contact rates (DUSCHER et al., 2005).

Zoonoses from wildlife represent the most significant, growing threat to global health of all emerging infectious diseases (JONES et al., 2008).

A close monitoring is necessary to plan and carry out control measures to prevent excessive transmission from wildlife reservoirs to humans and their pets. There is a significant lack of knowledge on many pathogens, vectors as well as reservoir hosts, which has to be filled by using new molecular methods and population modelling tools in the near future (DUSCHER et al., 2015).

Little information exists regarding the role of the aoudad as a pathogen reservoir. Furthermore, in most epidemiological surveys the potential role of coinfections (e.g. a first infection may make the host more immuno-competent or susceptible against a second pathogen) as a risk factor is often neglected. Compared to other wild ungulates in Spain, aoudads have high prevalence of antibodies against *M. bovis* (free = 49.5%; captive = 8%), very high prevalence of antibodies against *M. avium* subsp. paratuberculosis (free = 19.4%; captive = 56%), and intermediate prevalence of antibodies against *Salmonella* spp. (free = 13.4%; captive = 0%) or *Toxoplasma gondii* (free = 1.5%; captive = 24%). The wildlife managers must pay more attention to the potential risk posed by aoudads as hosts (and probably reservoirs) of paratuberculosis and tuberculosis mycobacterials, while epidemiologists should be more aware of coinfection as an important factor in epidemiological surveys, especially in wildlife populations where multiple infections are common (CANDELA et al., 2009).

Through this study it was verified that some pathogens are common to aoudads, domestic animals and to the human being. Then the occurrence of these wild animals in the

same range as domestic animals and humans can provide an excellent reservoir potential for some pathogens spread of diseases.

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Table 1. Hematologic values in captive Aoudad (*Ammotragus lervia*), 6 adults, (PEINADO et al., 1999).

Parameter	**Traditional Units**	**SI Units**
RBC - Red blood cells count	11.21-15.19 ($x10^6$ cells/ml)	11.21-15.19 ($x10^{12}$/L)
Hb - Hemoglobin	10.4-15.4 (g/dl)	104-154 (g/L)
PCV- Packed cell volume, Hematocrit	25.8-40.8 (%)	0.25-0.40 (L/L)
MCV- Mean corpuscular volume	22.7-27.7 (μm^3)	22.7-27.7 (fL)
MCH – Mean corpuscular hemoglobin	9.03-10.55 (pg/cell)	9.03-10.55 (pg/cell)
MCHC – Mean corpuscular hemoglobin concentration	35.8-42.4 (%)	358-424 (g/L)
WBC - White blood cells	2.12-5.28 ($x10^3$ cells/ml)	2.12-5.28 ($x10^9$/L)
Neutrophils	44.33-59.57 (%)	-
Neutrophils	1.02-2.80 ($x10^3$ cells/ml)	1.02-2.80 ($x10^9$/L)
Lymphocytes	37.12-49.78 (%)	-
Lymphocytes	0.94-2.30 ($x10^3$ cells/ml)	0.94-2.30 ($x10^9$/L)
Eosinophils	0.32-6.88 (%)	-
Eosinophils	0.06-0.26 ($x10^3$ cells/ml)	0.06-0.26 ($x10^9$/L)
Monocytes	0.00-1.99 (%)	-
Monocytes	0.00-0.12 ($x10^3$ cells/ml)	0.00-0.12 ($x10^9$/L)
Basophils	0.00-0.00 (%)	-
Basophils	0.00-0.00 ($x10^3$ cells/ml)	0.00-0.00 ($x10^9$/L)

Table 2 - Virus, Bacteria, Protozoa, Helminth and Ectoparasites in captive aoudad (*Ammotragus lervia*)

Pathogens	Status of animal	Samples/Method	Number/ Prevalence	Country	Reference
Virus					
Bluetongue virus (BT) antibodies	Healthy	Serum/ AGID and ELISA	6/17 (35.3%) 7/17 (41.2%)	Brazil	MORIKAWA, 2014
Bovine adenoviruses -1 antibodies	Healthy	Serum /VNT	5/11 (4.4 %)	Turkey	YEŞILBAĞ et al., 2011
Bovine adenoviruses -3 antibodies	Healthy	Serum /VNT	8/11 (72.7%)	Turkey	YEŞILBAĞ et al., 2011
Bovine respiratory syncytial virus antibodies	Healthy	Serum /VNT	1/11 (9.0%)	Turkey	YEŞILBAĞ et al., 2011
Malignant Catarrhal Fever (MCF)	Sick	Blood/PCR	1 animal	Israel	YERUHAM et al., 2004
Bacteria					
Campylobacter jejuni	Healthy	Feces/ Bacteriology	1/17 (5.8%)	Brazil	MORIKAWA, 2014
Chromobacterium violaceum	Sick – death	Lung parenchyma/ Histopathology and Bacteriology	1 animal	Spain	CARRASCO, 1996
Clostridium sp.	Sick – death	Lung parenchyma/ Bacteriology	1 animal	Australia	PORTAS et al., 2009
Enterococcus faecalis	Sick – death	Lung parenchyma/ Bacteriology	1 animal	Australia	PORTAS et al., 2009
Escherichia coli	Sick – death	Lung parenchyma/ Bacteriology	1 animal	Australia	PORTAS et al., 2009
Fusobacterium necrophorum	Death	Lesion/NI	2 animals	Korea	CHO et al., 2006
Klebsiella ferrigena	Sick – death	Lung parenchyma/ Bacteriology	1 animal	Australia	PORTAS et al., 2009
Leptospira spp. antibodies	Healthy	Serum /MAT	4/17 (23.5%)	Brazil	MORIKAWA, 2014
Mycobacterium avium	Death	Lung parenchyma/ Bacteriology	1 animal	Australia	PORTAS et al., 2009
Mycobacterium intracellulare	Sick – death	Bronchial lymph node/ Bacteriology	1 animal	Australia	PORTAS et al., 2009

Pathogens	Status of animal	Samples/Method	Number/ Prevalence	Country	Reference
Mycobacterium parafortuitum	Death	Mesenteric lymph node/ Bacteriology and PCR	1 animal	Australia	PORTAS et al., 2009
Mycobacterium tuberculosis	Death	Lung/Qpcr	1 animal	Brazil	MORIKAWA, 2014
Mycobacterium avium subsp. *paratuberculosis* antibodies	Death	Serum/EIA kit test	14/25 (56.0%)	Spain	CANDELA et al., 2009
Mycobacterium bovis antibodies	Death	Serum/modified indirect competition-EIA technique	2/25 (8.0%)	Spain	CANDELA et al., 2009
Mycoplasma mycoides subsp. *Mycoides*	Death	NI/ Bacteriology	1/15 (6.66%)	Germany	BRACK, 1966 apud SMITH et al., 1980
Salmonella spp. antibodies	Death	Serum/Agglutination test	9/67 (13.4%)	Spain	CANDELA et al., 2009
Protozoa					
Balantidium coli	Death	lymphatic ducts of the gastric lymph node and the abdominal mucosa/ Histopathology	1 animal	Korea	CHO et al., 2006
Besnoitia sp.	Death	Bronchial lymph node/ morphological examination	1animal	Australia	PORTAS et al., 2009
Cryptosporidium parvum mouse genotype	NI	Feces /IFT and PCR	1animal	China	KARANIS, 2007
Eimeria sp.	Sick – death	jejunum and ileum/ NI	1animal	Spain	CARRASCO, 1996
Neospora caninum antibodies	Healthy	Serum/ IFAT	4/17 (23.5%)	Brazil	MORIKAWA et al., 2014
Toxoplasma gondii antibodies	Healthy	Serum/IFAT	8/20 (40.0%)	Czech and Slovak	SEDLÁK et al, 2006
Toxoplasma gondii antibodies	Healthy	Serum/ IFAT	4/17 (23.5%)	Brazil	MORIKAWA et al., 2014
Toxoplasma gondii antibodies	Death	Serum/ MAT*	1/67 (1.5%)	Spain	CANDELA et al., 2009

Pathogens	Status of animal	Samples/Method	Number/ Prevalence	Country	Reference
Helminth					
Camelostrongylus mentulatus	Healthy	Abomasum/MCAM	10/19 (52.6%)	Spain	MAYO et al., 2013
Haemonchus contortus	Healthy	Abomasum/MCAM	1/19 (5.3%)	Spain	MAYO et al., 2013
Marshallagia marshalli	Healthy	Abomasum/MCAM	4/19 (21.1%)	Spain	MAYO et al., 2013
Nematodirus abnormalis	Healthy	Small intestine/MCAM	3/22 (13.6%)	Spain	MAYO et al., 2013
Nematodirus filicollis	Healthy	Small intestine/MCAM	2/22 (9.1%)	Spain	MAYO et al., 2013
Nematodirus helvetianus	Healthy	Small intestine/MCAM	3/22 (13.6 %)	Spain	MAYO et al., 2013
Nematodirus spathiger	Healthy	Small intestine/MCAM	3/22 (13.6%)	Spain	MAYO et al., 2013
Ostertagia leptospicularis	Healthy	Abomasum/MCAM	1/19 (5.3%)	Spain	MAYO et al., 2013
Ostertagia lyrata	Healthy	Abomasum/MCAM	1/19 (5.3%)	Spain	MAYO et al., 2013
Ostertagia ostertagi	Healthy	Abomasum/MCAM	1/19 (5.3%)	Spain	MAYO et al., 2013
Skrjabinema ovis	Healthy	Large intestine/MCAM	9/23 (39.1%)	Spain	MAYO et al., 2013
Teladorsagia circumcincta	Healthy	Abomasum/MCAM	10/19 (52.6%)	Spain	MAYO et al., 2013
Teladorsagia trifurcata	Healthy	Abomasum/MCAM	1/19 (5.3%)	Spain	MAYO et al., 2013
Trichostrongylus capricola	Healthy	Small intestine/MCAM	1/22 (4.5%)	Spain	MAYO et al., 2013
Trichostrongylus colubriformis	Healthy	Small intestine/MCAM	8/22 (36.4%)	Spain	MAYO et al., 2013
Trichostrongylus probolorus	Healthy	Small intestine/MCAM	3/22 (13.6%)	Spain	MAYO et al., 2013
Trichostrongylus vitrinus	Healthy	Small intestine/MCAM	8/22 (36.4%)	Spain	MAYO et al., 2013
Trichuris spp.	Healthy	Large intestine/MCAM	2/23 (8.7%)	Spain	MAYO et al., 2013
Ectoparasites					
Arthropod					
Sarcoptes scabiei	Sick	Skin scrapings/light microscopic	1 animal	Israel	YERUHAM et al., 1996

AGID - Agar Gel Immunodiffusion; ELISA - Enzyme Linked Immunosorbent Assay; IFT - Immunofluorescence test ; IFAT - Indirect fluorescence antibody test; qPCR - Real-Time Polymerase Chain Reaction ; MAT - Microscopic Agglutination Test; MAT* - Modified direct agglutination test incorporating 2-mercaptoethanol and formalin-fixed tachyzoites; MCAM - Morphometric characteristics of adult male; NI - Not Informed; VNT - Virus neutralization test.

Table 3 - Virus, Bacteria, Protozoa, Helminth and Ectoparasites in free-living aoudad (*Ammotragus lervia*)

Pathogens	Status of animal	Samples/Method	Number/ Prevalence	Country	Reference
Virus					
Bluetongue (BT) antibodies	Healthy	Serum /Official Modified Compliment Fixation test, BT Agar Gel Immunodiffusion test.	11/12 (91.66%)	USA	HAMPY et al., 1979
epizootic hemorrhagic disease (EHD) antibodies	Healthy	Serum /Compliment Fixation test	12/12 (100%)	USA	HAMPY et al., 1979
infectious bovine rhinotracheitis (IBR) antibodies	Healthy	Serum /Modified Direct Compliment Fixation test	9/12 (75.0%)	USA	HAMPY et al., 1979
Bacteria					
Chlamydiaceae antibodies	Healthy	Serum/ELISA reactions LPS antigen	5/14 (35.71%)	Spain	SALINAS et al., 2009
Chlamydophila abortus antibodies	Healthy	Serum/ELISA reactions POMP antigen	1/14 (7.14%)	Spain	SALINAS et al., 2009
M. avium subsp. *paratuberculosis* antibodies	Death	Serum /EIA kit test	13/67 (19.4%)	Spain	CANDELA et al., 2009
Mycobacterium bovis antibodies	Death	Serum/modified indirect competition-EIA technique	33/67 (49.25%)	Spain	CANDELA et al., 2009
Protozoa					
Neospora caninum antibodies	Death or captured	Serum/ ELISA and IFAT	1/13 (7.7%)	Spain	ALMERÍA et al., 2007
Toxoplasma gondii antibodies	Death	Serum/ MAT*	6/25 (24.0%)	Spain	CANDELA et al., 2009
Toxoplasma gondii antibodies	NI	Serum/ MAT	1/10 (10.0%)	Spain	GAUSS et al., 2006
Helminth					
Elaeophora schneideri	Death	carotid artery / macroscopic observations	3/9 (33.3%)	USA	PENCE & GRAY, 1981

Pathogens	Status of animal	Samples/Method	Number/ Prevalence	Country	Reference
Ectoparasites					
Tick					
Dermacentor albipictus	NI	NI	NI	USA	GRAY,1979
Otobius megnini	NI	NI	NI	USA	GRAY,1979
Louse					
Bovicola neglecta	NI	NI	NI	USA	GRAY, 1979
Bovicola sp.	NI	NI	NI	USA	GRAY, 1979
Arthropod					
Sarcoptes scabiei	NI	skin scrapings / light microscopic	43/342 (12.6%)	Spain	GONZÁLEZ-CANDELA et al., 2004

ELISA - enzyme linked immunosorbent assay; IFAT - indirect fluorescent antibody test; MAT - Microscopic Agglutination Test; MAT* - Modified direct agglutination test incorporating 2-mercaptoethanol and formalin-fixed tachyzoites; NI- Not Informed.

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SUPPLEMENT 2

7.2 MOLECULAR SCREENING OF *Plasmodium* SPECIES IN CAPTIVE CERVIDS IN SOUTHERN BRAZIL

ABSTRACT

The epidemiology of *Plasmodium* spp. infections in primates is extensively studied, however few studies have been conducted in ungulates, including deer. Indeed, almost half a century has gone with no other description of *Plasmodium* spp. in deer since its first identification in the Americas in 1967. Recently, however, we have seen a rediscovery of *Plasmodium* research in ungulates reporting the role of deer as reservoirs of malaria parasite in the USA. Accordingly, we have evaluated a captive herd of 22 Brazilian dwarf brocket deer (*Mazama nana*), four red brocket deer (*Mazama americana*) and six marsh deer (*Blastocerus dichotomus*) from southern Brazil; by using light microscopy and molecular approaches. Extracted DNA samples were tested for amplified fragments of both *Plasmodium* spp. genes, mitochondrial cytochrome b (*cytb*) and small subunit ribosomal RNA (SSU rRNA). Microscopic and molecular analyses were both negative for parasite presence. Since all samples were obtained in a single time point collection and parasitemia may fluctuate in different stages, monitoring and/or multiple sequential sampling should be further addressed to insure absence of infection and disease. This study was part of an active surveillance work to monitor the health status of captive cervids.

Key-words: Brazil, cervids, deer, malaria, molecular screening, *Plasmodium*.

DEVELOPMENT

Malaria parasites have been described in a wide range of hosts in the Americas, including humans beings (CARTER et al., 2015), monkeys (ARAÚJO et al., 2013), free-living birds (CHAGAS et al., 2016), reptiles (MOTZ et al., 2014) and rodents (dos SANTOS et al., 2009). Among non-primate mammals, *Plasmodium* species had been thought to be limited to the Old World and in particular, cervids had not been considered as a vertebrate host due to the absence of parasites during blood smear investigations (GARNHAM & KUTTLER, 1980; DAVIDSON et al., 1983; DAVIDSON et al., 1985). This idea was proved wrong in 1967 with the identification of a *Plasmodium* parasite (named *P. odoicoleus*) in a blood smear of a splenectomized white-tailed deer (*Odoicoleus virginianus*) from Texas, USA (KUTTLER et al., 1967; GARNHAM & KUTTLER, 1980).

Not surprisingly, recent reports have “rediscovered” malaria parasites in cervids and other ungulates throughout the world (BOUNDENGA et al., 2016; MARTINSEN et al., 2016; TEMPLETON et al., 2016). These studies have raised questions on the evolution of *Plasmodium* parasites, the parasite cross-continental dispersion and the role of ungulates as malaria reservoirs. The identification of a *Plasmodium*-positive deer in North America lead us to question if the presence of malaria reservoirs extends to the South American cervids. A large herd of captive Brazilian dwarf brocket deer (*Mazama nana*) in Brazil is protected by the Bela Vista Biological Sanctuary (BVBS, Foz do Iguaçu, Brazil), which also keeps red brocket deer (*M. americana*) and marsh deer (*Blastocerus dichotomous*). BVBS (25º 26' 57" S, 54º 33' 18" W) is a zoo and rehabilitation animal center located in a national protected area in South Brazil, sharing borders with Argentina and Paraguay. Cervids are kept in fenced areas covered and surrounded by native vegetation, making the contact with free-living wild animals possible. Capybaras (*Hydrochaeris hydrochaeris*) are often seen moving freely by the zoo areas. Our research group have first reported *Plasmodium* sp. infection in capybaras, which were captive animals at the BVBS, with 1/11 on microscopy and 3/11 on molecular testing (dos SANTOS et al., 2009).

In addition, the mosquito *Anopheles* sp., implicated vector for the *Plasmodium* spp. transmission, has been shown a >10% prevalence on the region, favored by the humid and warm climate (FALAVIGNA-GUILHERME et al., 2005). Altogether, this region may provide enough conditions for a complete transmission cycle to wildlife mammals, particularly cervids.

In Brazil, Brazilian dwarf brocket deer, red brocket deer and marsh deer have been screened for pathogens such as hemotropic mycoplasmas (hemoplasmas), toxoplasmosis, brucellosis and neosporosis (GRAZZIOTIN et al, 2011; ZIMPEL et al., 2015). Such monitoring has provided important information for animal and public health, and conservation aspects, since marsh deer have been considered internationally vulnerable (APRIL & DUARTE, 2008), while dwarf and red brocket deer have been described as vulnerable species in this country (DUARTE et al., 2012a; DUARTE et al., 2012b).

Blood samples from 32 cervids previously surveyed for other pathogens (GRAZZIOTIN at al., 2011a; ZIMPEL et al., 2015) were included in this study. Complete blood count (CBC) was performed, and peripheral blood smears were stained with May-Grünwald-Giemsa stain and examined using light microscopy at 1,000x (BX51, Olympus, Tokyo, Japan).

DNA was extracted using a commercial kit (Dneasy Blood & Tissue Kit, Qiagen, Valencia, California, USA), according to manufacturer's instructions. DNA quality was assessed by amplification of the mitochondrial cytochrome b (cytb) gene (KOCHER et al., 1989), using primers L14841 (5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3') and H15149 (5'-AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA-3'). Molecular screening of *Plasmodium* spp. was performed using two previously described protocols, one targeting the small subunit RNA gene (SSU rRNA) (dos SANTOS et al., 2009; BUENO et al., 2010) and the other targeting the mitochondrial cytocrome b gene (*cytb*) of the parasite (MARTINSEN et al., 2006; MARTINSEN et al., 2016). All reactions were performed using positive and negative controls.

No *Plasmodium*-like parasite was observed by direct examination of deer blood smears, and no DNA amplification was detected by PCR on either SSU rRNA or *cytb* genes. Despite these negative results at this pinpoint sampling, results of both methods cannot ensure that animals are free of *Plasmodium* sp. infection. Low parasitemia has been previously described for infected ungulates (GARNHAM & KUTTLER, 1980; MARTINSEN et al., 2016; TEMPLETON et al., 2016), such as low as 0.003% (TEMPLETON et al., 2016) in molecular tests with no detection of parasites in blood smears. The life cycle of *Plasmodium* spp. in the vertebrate host may include a long-lived dormant stage in the liver, with sequestration from general circulation causing low parasitemia, as previously reported in water buffalos (*Bubalus bubalis*) in the absence of an immunosuppressed state (SHEATHER, 1919).

The nested PCR-based screening of white tailed deer from 45 counties in the United States has found 41/308 (13.3%) animals infected by *Plasmodium* sp., all from the eastern region of the country. Conversely, previous studies have failed in detect *Plasmodium* in other ungulate species, including elk (*Cervus canadensis*), pronghorn (*Antilocapra americana*) and mule deer (*Odocoileus hemionus*) (MARTINSEN et al., 2016). Additionally, previous PCR-based screening studies with other species of the Order Artiodactyla, including sitatunga (*Tragelaphus spekei*), red river hog (*Potamochoerus porcus*) and water chevrotain (*Hyemoschus aquaticus*) have also not detected *Plasmodium* sp. (BOUNDENGA et al., 2016).

Therefore, a potential parasite life cycle during the time of blood collection might have had direct impact to the negative findings. In addition, recent molecular clock estimates of the *Plasmodium* spp. divergence have showed that the clade including *P. odoicoleus* likely diverged from other clades between 2.3 and 6 million years ago (MARTINSEN et al., 2016),

suggesting that it is an ancient parasite of deer. Thus, co-evolution of both species could have make deer a well adapted host for the parasite. Other previous investigations of *Plasmodium* spp. in deer have failed to detect the parasite (GARNHAM & KUTTLER, 1980; DAVIDSON et al., 1983; DAVIDSON et al., 1985) and some *Plasmodium* species have been discovered only after having a host splenectomized (KUTTLER et al., 1967).

The global health and ecological impact of malaria in wild animals is still unknown. Therefore, active surveillance, providing epidemiological information regarding health status, mortality rates, geographic distribution of malaria infection in wild (free and captive animals) and domestic ungulates, including deer, is important to aid the understanding of parasite evolution and strategies in disease management and control, in addition to promote sustained animal well-being.

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