UNIVERSIDADE FEDERAL DO PARANÁ

RAFAEL FELIPE DA COSTA VIEIRA

DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE HEMOPLASMA EM CAPIVARAS *(Hydrochaeris hydrochaeris)* DE VIDA LIVRE E CATIVEIRO EM FOZ DO IGUAÇU, PARANÁ

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Orientador: Prof. Dr. Marcelo Beltrão Molento

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A Comissão Examinadora da Defesa da Dissertação intitulada **"DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE HEMOPLASMA EM CAPIVARAS** *(Hydrochaeris hydrochaeris)* **DE VIDA LIVRE E CATIVEIRO EM FOZ DO IGUAÇU, PARANÁ"** apresentada pelo Mestrando RAFAEL FELIPE DA COSTA VIEIRA declara ante os méritos demonstrados pelo Candidato, e de acordo com o Art. 78 da Resolução nº 62/03–CEPE/UFPR, que considerou o candidato APTO para receber o Título de Mestre em Ciências Veterinárias, na Área de Concentração em Ciências Veterinárias.

Curitiba, 26 de maio de 2009.

Prof. Dr. Marcelo Beltrão Molento
Presidente/Orientador

Prof. Dr. Odilon Vidotto
Membro

Dr. Stenio Perdigão Fragoso
Membro

À minha amada mãe Maria Marilene da Costa,
responsável por todas as minhas conquistas pessoais
e profissionais. Sua incansável luta sempre buscando meu bem,
deu-me forças para continuar seguindo minha caminhada
em busca da felicidade. Espero estar respondendo à altura
de suas espectativas. Obrigado por ter me tornado um
homem que não foge a luta!

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Notas de Cada Dia

Convece-te de que não existem males eternos.
Toda dor chega e passa.
O dia é sempre novo para quem trabalha.
A dificuldade é uma escola.
Servir é um privilégio.
Auxilia para o bem.
Nada reclames.
A paciência operosa realiza prodígios.
E, à frente de quaisquer desenganos,
não te esqueças de que o tempo de hoje continuará amanhã.

Emmanuel

RESUMO

Os micoplasmas hemotrópicos são bactérias gram negativas, em forma de cocos, anéis ou bacilos, que parasitam eritrócitos de uma grande variedade de mamíferos, entre eles os roedores, e podem causar uma enfermidade conhecida como hemoplasmose. No Brasil, vários estudos foram realizados em diversas regiões envolvendo diferentes espécies. Os sinais da doença incluem febre, anorexia, mucosas pálidas e a anemia, quando presente, é discreta a moderada. Em roedores é causada pelas bactérias *Mycoplasma coccoides* e *M. haemomuris*. A transmissão ocorre pela picada do piolho *Polyplax serrata* e *P. spinulosa*, transfusão sanguínea e via transplacentária. O diagnóstico é realizado pela visualização do agente infeccioso sobre a membrana do eritrócito ao exame do esfregaço sanguíneo. A reação em cadeia da polimerase (PCR) é considerada o padrão ouro por apresentar maior sensibilidade e especificidade que a citologia. Apesar de serem relatadas várias espécies de roedores acometidos por *Mycoplasma* sp., a capivara (*Hydrochaeris hydrochaeris*) não havia sido testada. Amostras de sangue de 31 capivaras: 10 de cativeiro e 21 de vida livre foram coletadas. O DNA foi extraído e a PCR foi realizada para detecção do gene 16S rRNA do *M. coccoides* e *M. haemomuris*. Usando o protocolo do *M. coccoides* 20/31 (64%) das capivaras foram positivas, incluindo 17/21 (80%) de vida livre e 3/10 (30%) de cativeiro. A diferença da prevalência da infecção entre os grupos foi considerada significativa (p = 0,001). O sequenciamento de quase todo o gene 16S rRNA de amostras positivas sugerem um novo isolado de hemoplasma com identidade de 92% com o *M. coccoides* e 86% com o *M. haemomuris*. Todas as amostras de capivaras foram negativas a infecção pelo *M. haemomuris*. O DNA do gene constitutivo GAPDH foi amplificado em todas as amostras. Esta é a primeira descrição de infecção por hemoplasmas em capivaras.

Palavras-chave: Capivara (*Hydrochaeris hydrochaeris*). Micoplasmas hemotrópicos. PCR. *Mycoplasma coccoides. Mycoplasma haemomuris*.

ABSTRACT

Hemotropic mycoplasmas are gram negative, coccoid, ring or rod shaped bacteria, which parasite erythrocytes of a wide variety of mammals, including rodents, and can cause a disease named hemoplasmosis. In Brazil, many studies were performed in a wide of regions involving different species. Signs of disease include fever, anorexia, pale mucous membranes and anemia, when present, is mild to moderate. In rodents is caused by *Mycoplasma coccoides* and *Mycoplasma haemomuris* bacteria. Transmission occur by the bite lice *Polyplax serrata* and *Polyplax spinulosa*, blood transfusion and transplacental. Diagnosis is made by visualization of infectious agent on the erythrocyte membrane over blood smear examination. Polymerase chain reaction (PCR) is considered the gold standard test due to higher sensitivity and specificity than cytology. Although a wide range of rodents species infected by *Mycoplasmas* sp. were described, capybara (*Hydrochaeris hydrochaeris*) had not been tested. Blood samples from 31 animals: 10 captive and 21 free-ranging capybaras were collected. DNA was extracted and PCR assays for the detection of the 16S rRNA from the *M. coccoides* and *M. haemomuris* were performed. Using the *M. coccoides*-PCR assay 20/31 (64%) of the capybaras were positive, including 17/21 (80%) free-ranging and 3/10 (30%) from captive animals. The prevalence of infection between the groups was significantly different (p = 0.001). Sequencing of the nearly entire 16S rRNA gene from the positive samples suggested a novel hemoplasma isolate with identity of 92% with *M. coccoides* and 86% with *M. haemomuris*. All capybara samples were negative for *M. haemomuris* infection. DNA of a GAPDH housekeeping gene was successfully amplified from all samples. This is the first evidence of a hemoplasma infection in capybaras.

Key words: Capybara (*Hydrochaeris hydrochaeris*). Hemotrophic mycoplasmas. PCR. *Mycoplasma coccoides. Mycoplasma haemomuris*.

SUMÁRIO

1 INTRODUÇÃO

Micoplasmas hemotróficos (hemoplasmas) são bactérias pequenas (0,3 a 2,0µm), pleomórficas, gram-negativas, sem parede celular, que aderem à membrana dos eritrócitos causando sinais clínicos variáveis, que vão desde uma anemia aguda a uma forma crônica sem alterações hematológicas evidentes. Já foram descritos em várias espécies de mamíferos incluindo cães, gatos, bovinos, suínos e até mesmo no homem (SANTOS *et al.,* 2008; GUIMARAES, 2008; GUIMARAES *et al*., 2007; TAGAWA *et al*., 2008; SANTOS *et al.*, 2008b). Fatores de risco como esplenectomia, imunossupressão e co-infecção envolvendo outros agentes podem desenvolver a infecção. Embora a visualização isolada ou em grupos destes organismos aderidos às hemáceas em esfregaços de sangue corados seja conhecida há décadas, estes ainda não são cultiváveis *in vitro*, o que limita muito os estudos experimentais (MESSICK, 2004).

O diagnóstico é baseado na visualização direta do agente em esfregaços sanquíneos é pouco sensível e pouco específico (HARVEY, 2006). A utilização da reação em cadeia da polimerase (PCR) é mais sensível e mais específica pois detecta o DNA no sangue dos animais.

Em roedores, as espécies já descritas são *Mycoplasma coccoides* e *Mycoplasma haemomuris* (WILLI *et al*., 2007; ZHANG; RIKIHISA, 2002). Porém, não havia relatos na literatura de infecção por hemoplasmas em capivaras, maior roedor do mundo. Assim, avaliou-se a presença de hemoplasmas em capivaras de cativeiro e vida livre do Refúgio Biológico Bela Vista, Itaipu Binacional.

2 HAEMOPLAMAS (HAEMOTROPHIC MYCOPLASMAS) IN BRAZIL

HEMOPLASMAS (MICOPLASMOSE HEMOTRÓFICA) NO BRASIL

ABSTRACT

Recent studies using molecular techniques for the detection of hemotrophic mycoplasmas in several mammals have been conducted in Brazil. In domestic cats, *Mycoplasma haemofelis*, '*Candidatus* M. haemominutum' and '*Candidatus* M. turicensis' infections have been identified. These species have also been found in free ranging and captive neotropical felid species. Two canine hemoplasmas, *Mycoplasma haemocanis* and '*Candidatus* Mycoplasma haematoparvum', have been identified in dogs. In commercial swine populations, *Mycoplasma suis* was found to be highly prevalent, especially in sows. Moreover, novel mycoplasma species have been identified in Brazilian commercial pigs and domestic dogs. A hemoplasma infection in a human patient infected with the human immunodeficiency virus was also recently documented. In conclusion, hemoplasma species are common and important infectious agents in Brazil. Further studies should be conducted to better understand their impact on pets, production animals and wildlife fauna, as well as their role as zoonotic agents, particularly in immunocompromised patients.

Key words: Hemoplasma. *Mycoplasma haemofelis. Mycoplasma suis.* Mycoplasmosis.

RESUMO

Estudos recentes utilizando técnicas moleculares para a detecção de micoplasmas hemotróficos em diferentes mamíferos têm sido conduzidos no Brasil. Em gatos domésticos, *Mycoplasma haemofelis*, '*Candidatus* M. haemominutum' e '*Candidatus* M. turicensis' foram identificados. Estas espécies também foram encontradas em felídeos neotropicais de vida livre e de cativeiro, Dois hemoplasmas caninos foram identificados em cães domésticos. Em suínos de produção, *Mycoplasma suis* possui alta prevalência, especialmente em porcas. Além disso, novas espécies de hemoplasmas foram detectadas em suínos comercias e cães. Recentemente, a infecção por hemoplasmas em um paciente humano portador do vírus da imunodeficiência humana foi documentada. Em conclusão, espécies de hemoplasmas são comuns e importantes agentes de infecções no Brasil. Estudos futuros devem ser conduzidos para melhor entender seu impacto em cães e gatos, animais de produção e na fauna silvestre, e também para determinar o seu papel como agentes zoonóticos, particularmente em pacientes imunocomprometidos.

Palavras-Chave: Hemoplasma. *Mycoplasma haemofelis*. *Mycoplasma suis.* Micoplasmose.

2.1 INTRODUCTION

Hemoplasmas (hemotrophic mycoplasmas) are extracellular, rod-shaped, small, pleomorphic bacteria that attach to the red blood cells of a wide range of mammalian species and may induce anemia in an infected host. These organisms have not been grown successfully in culture. Initially the hemoplasmas were classified in the order Rickettsiales based on morphology and response to antibiotic therapy (WEISS; MOULDER, 1984). However, molecular studies based on the16S rRNA gene have shown that the genera *Haemobartonella* and most *Eperythrozoon* are more closely related to the pneumoniae group of mycoplasmas, leading to the reclassification within the genus *Mycoplasma* (NEIMARK *et al*., 2001; NEIMARK *et al*., 2002).

The transmission of hemoplasmas occurs mainly by blood-sucking vectors; cats by fleas (LAPPIN *et al*., 2003); dogs by the tick *Rhipicephalus sanguineus* (SENEVIRATNA *et al*., 1973); and rodents by murine lices *Polyplax serrata* and *Polyplax spinulosa* (BERKENKAMP; WESCOTT, 1988) have been reported. Under experimental conditions, arthropod vectors, including lice, mosquitoes, and stable flies, can transmit *M. suis* infection to pigs (SMITH, 1986). Other means of transmission are suspected, such as fight episodes, blood transfusion, needles and fomites (HARVEY, 2006).

The host-adapted survival of the hemoplasmas is achieved through surface parasitism of the red blood cell. They depend on the host cell for provision of amino acids, fatty acids, cholesterol, and vitamins. The failure of hemoplasmas to grow in a defined medium is likely because of our inability to duplicate the complex nutritional support provided bu the host (MESSICK, 2004).

The disease often follows a chronic course with wide-ranging clinical signs. The acute infection is associated with mild to moderate hemolytic anemia, anorexia, fever and icterus, depending on the hemoplasma species involved. In dogs and pigs clinical signs of the disease are rare, but in immunocompromised and splenectomised patients they are presents. Differences in the severity of

clinical disease may represent differing pathogenicity of hemoplasmas (MESSICK, 2004).

Hemoplasmas have been observed in all continents but Antarctica. There appears to be a high prevalence of hemotrophic mycoplasmas in tropical regions, likely due to environmental conditions favoring transmission of hemoplasmas by vector-borne agents (MACIEIRA, 2008). Over the last few years, new hemoplasma species have been described in Brazilian pigs (MESSICK *et al*., 2007) and dogs (SANTOS *et al*., 2008a). In addition, cross-species infection has also been reported (NEIMARK *et al*., 2004). Recently, a hemoplasma infection in a HIV-positive human patient was described (SANTOS *et al*., 2008b). Our goal in this review is to summarize all recent molecular studies that characterize the occurrence of hemoplasmas in Brazil (table 1).

2.1.1 Companion Animals

Although *Mycoplasma* sp. have been frequently found on blood smears of cats in Brazil, molecular approaches for detecting hemotrophic mycoplasma species have increased the detection sensitivity in anemic and non anemic cats from different regions of Brazil. The three currently known species of feline hemoplasmas (formerly known as a single species, *Haemobartonella felis*) have been recently described in Brazil (MORAIS *et al*., 2007; GUIMARAES, 2008; SANTOS, 2008).

Using PCR, 14 of 37 (38%) anemic cats (PCV < 24%), from a veterinary hospital of Curitiba harbored *M. haemofelis,* 4 (11%) were positive for '*Candidatus* M. haemominutum', and 3 (8%) were co-infected with both organisms (BAUMANN *et al*., 2006). Blood smear evaluations in this study were not reliable for hemoplasma diagnosis. Mycoplasma-like bodies were found in only about 1/3 of PCR positive samples.

In Porto Alegre 371 cats were tested by PCR for the 3 feline hemoplasmas;

21.3% were infected by at least one species. The most prevalent species in this study was '*Candidatus* M. haemominutum' with 50 positive cats (13.5%), whereas '*Candidatus* M. turicensis' and *M. haemofelis* were found in 10 (2.7%) and 8 (2.2%) cats, respectively (SANTOS, 2008). Male cats and cats with outdoor access were more likely to be infected with hemoplasmas in this study. This was also the first report of '*Candidatus* M. turicensis' in domestic cats in Brazil.

In the cats presented in the Small Animal Hospital at University of São Paulo, in Southeast Brazil, *M. haemofelis* was found in 23 cats (8.5%) of 270 anemic cats (PCV < 30%) (HORA, 2008). Another survey compared 80 healthy and 74 sick cats from Botucatu (São Paulo state); eight (10%) healthy cats and 15 (20%) of the symptomatic cats were positive for *M. haemofelis* and/or '*Candidatus* M. haemominutum' (BATISTA, 2004). Of the 23 positive samples, 11 had *M. haemofelis*; 4 *M. haemominutum* and 8 were co-infected with both organisms. Association between clinical signs and infection with *M. haemofelis* was observed, suggesting a higher virulence of *M. haemofelis* when compared to *M. haemominutum*. One hundred and forty-nine cats from Rio de Janeiro were also screened for hemoplasmas using PCR and southern blot; fifteen (10%) were positive for '*Candidatus* M. haemominutum', 6 (4%) for *M. haemofelis*, and 3 (2%) were co-infected with both hemoplasmas (MACIEIRA *et al*., 2006; MACIEIRA *et al*., 2008).

Results were higher than frequency found also in Rio de Janeiro by blood smear evaluation. There appears to be a variation in the prevalence of feline hemoplasmosis in different cities from the South and Southeast regions of Brazil. Hemoplasma infection in anemic cats ranged from 8.5% in São Paulo to 38% in Curitiba. Prevalence in samples including anemic and non-anemic cats showed an overall prevalence of 16% in Rio de Janeiro and 15% in Botucatu. Cats infected with feline immunodeficiency virus (FIV), either alone or in association with feline leukemia virus (FeLV) were at a higher risk of harboring '*Candidatus* M. haemominutum' than retroviral negative cats in studies in Rio de Janeiro and São Paulo (HAGIWARA *et al*., 1997; HORA, 2008; MACIEIRA *et al*., 2008).

It is impossible to directly compare the percentage of hemoplasma-infected cats reported in these various studies. The problem is that the occurrence of infection is reported in different cat populations. The fact that not all hemoplasmas are evaluated in every study further confounds our ability to correlate these results. Nonetheless, it can be concluded that hemoplasma infections are common in cats from Southern Brazil.

Dogs are known to be infected with at least two hemoplasmas, *M. haemocanis* and '*Candidatus* M. haematoparvum'. *Mycoplasma haemocanis* was described in blood smears of dogs from Belo Horizonte (19^{o} 49' 01" S, 43^{o} 57' 21" W) (BIONDINI, 1983; O'DWYER *et al*., 1997), later characterized by PCR in four non-splenectomized dogs from a veterinary hospital of Londrina (23^{o} 18' 36" S, 51^{o} 09' 46" W) (MORAIS *et al.*, 2003) and other dog populations (SANTOS *et al*., 2008a; TRAPP *et al*., 2006). Recently, a PCR survey was conducted in Belo Horizonte and the surroundings to compare infection rates for *M. haemocanis* between urban and rural areas (SANTOS *et al*., 2008a). Twenty of 176 (11.3%) dogs living in rural areas were positive, whereas 6 of 104 (5.8%) dogs from urban areas harbored the organism. Although a survey on 78 healthy blood donors in São Paulo showed no positive sample for *M. haemocanis*, only blood smear evaluation was made and therefore results were not reliable (MOREIRA *et al*., 2007).

A PCR survey for both *M. haemocanis* and '*Candidatus* M. haematoparvum' was conducted in Londrina (SANTOS *et al*., 2008a). A total of 147 dogs with at least one laboratory abnormality (leucopenia, thrombocytopenia, pancytopenia or anemia) were tested, resulting in 2 (1.4%) positive samples for *M. haemocanis* and 11 (7.5%) for '*Candidatus* M. haematoparvum'. However, the sequenced PCR amplicons of '*Candidatus* M. haematoparvum' was only 85% similar to '*Candidatus* M. haematoparvum' (EF416569), but showed 96% homology to *M. haemofelis* and 96% to *M. haemocanis*. Results suggested that, although amplified by a standard '*Candidatus* M. haematoparvum' PCR protocol, this particular species of

hemoplasma is phylogenetically closer to the large hemoplasmas of dogs and cats than to '*Candidatus* M. haematoparvum'.

In addition, two other novel hemoplasmas have been identified by PCR in dogs from Brazil (TRAPP *et al*., 2006; SANTOS *et al*., 2008a). A small canine hemoplasma more similar to '*Candidatus* M. haemominutum' than '*Candidatus* M. haematoparvum' was detected by PCR in a dog from Londrina (SANTOS *et al*., 2008a), and a novel hemoplasma with 98-100% homology with the '*Candidatus* M. turicensis' found in cats was also amplified in 7 of 10 splenectomized dogs from Porto Alegre (SANTOS et al., 2008a). It is not clear if this is a new canine hemoplasma or a demonstration that '*Candidatus* M. turicensis' also can infect dogs.

2.1.2 Production Animals

Mycoplasma suis was detected by PCR and Southern blot in 186 pigs (121 sows, 61 piglets and 4 boars) from four farms in Southern Brazil, the region responsible for most of the pork production in the country. In this study, 22 sows (18.2%) were positive by PCR, whereas 40 (33.1%) were positive by Southern blot; one piglet (2%) and one of four boars were also positive. There was no association between packed cell volume and the infection, suggesting a sub clinical form of the disease in pigs (GUIMARAES *et al*., 2007a). Therefore, although acute infection and high mortality rates have declined due to the addition of tetracycline to the food, *M. suis* infection is still present in pig herds and may adversely affect weight gain and meat production. In addition, a novel mycoplasma species was detected in pigs from these farms (MESSICK *et al*., 2007); 14 (7.5%) of 186 domestic pigs were PCR-positive, some of them coinfected with *M.suis*. When first described in 1950s, eperythrozoonosis was a disease known to have two distinct causative agents: *E. parvum* and *E. suis*, however, pigs infected with *E. parvum* showed few clinical signs. This parasite has not yet been fully characterized, except for three

reports more than 40 years ago and another description more than 25 years ago (SPLITTER, 1950; SPLITTER, 1953; NEIMARK *et al*., 2005). The novel species characterized in this study is likely to be the putative parasite *E. parvum*.

Although hemoplasma species have not been described in Brazilian ruminants, they are likely present. *Mycoplasma wenyonii*, the hemoplasma classically associated with infection in cattle, has been described in United States, Japan, Venezuela, Switzerland, and England (SMITH *et al*., 1990; BRETANA *et al*., 2002; HOFMANN-LEHMANN *et al*., 2004; MCAULIFFE *et al*., 2006; RIOND *et al*., 2007; TAGAWA *et al*., 2008). A new species, '*Candidatus* Mycoplasma haemobos' was also recently identified in bovine from Japan (TAGAWA *et al*., 2008).

2.1.3 Wildlife Animals

The three species of feline hemoplasmas have been found in both free ranging and/or captive wild cat species (WILLI *et al*., 2007). In a study with 110 captive wild felids, including five non-native species, 11 were positive for '*Candidatus* M. haemominutum' (1 lion, 1 Geoffroy's cat, 1 margay, 4 ocelot, 3 oncilla and 1 puma), 2 were positive for *M. haemofelis* (1 margay and 1 ocelot), and 1 was positive for '*Candidatus* M. turicensis' (1 Ocelot). In another study, a 12 year old lion was PCR positive for '*Candidatus* M. haemominutum' at the Zoological Garden of Curitiba, Southern Brazil (GUIMARAES *et al*., 2007b). Recently, a total of 57 neotropical felids maintained in captivity at Foz do Iguaçu in Southern Brazil were tested for *M. haemofelis* and '*Candidatus* M. haemominutum' infection by PCR. Only a 7 year-old margay showed a positive result for *M. haemofelis*. It is of particular interest that flea infestation is more common in São Paulo Zoos (ADANIA *et al*., 1998) and uncommon in Refúgio Bela Vista at Foz do Iguaçu (GUIMARAES, 2008). This may explain the differences in hemoplasma infection reported in these studies.

In Marana and Clara, Northeastern Brazil, another survey in 29 geotropically felids was evaluated. Six ocelots (*Leopardus pardalis*) and one jaguarondi (*Puma yagouarondi*) were positive (24.1%). Three of them were co-infected with *M. haemofelis* and '*Candidatus* M. haemominutum' (two ocelots and one jaguarondi), and four only with '*Candidatus* M. haemominutum' (SANTOS *et al*., 2008a).

A search for blood parasites in 374 small wild mammals, including nine rodent (*Macedon montensis*, *A. serrensis*, *Delomys dorsalis*, *D. sublineatus*, *Nectomys squamypes*, *Oligorysomys nigripes*, *Oxymycterus* sp, *Thaptomys nigrita*, and *Trynomis dimediadus*) and six marsupial (*Didelphis aurita*, *Gracilynanus* sp, *Marmosops incanus*, *Monodelphis scallops*, *Philander frenata*, *Thylamis velutinus*) species was undertaken in three areas of the Atlantic Forest in Southeastern Brazil (SILVA *et al*., 2007). A total of 65 specimens (17.4%) belonging to 9 different mammals (6 rodents and 3 marsupials) were infected with hemotrophic mycoplasmas or *Babesia* sp. Packed cell volume and total plasma protein were similar between infected and non-infected specimens suggesting that wild mammals may have a sub-clinical or chronic infection. Other native Brazilian wild species may also be infected with hemotrophic mycoplasmas, and further studies should be conducted to establish animal reservoirs, animal hosts and novel hemoplasma species. *Mycoplasma* spp. detection in squirrel monkeys (*Saimiri sciureus*) in French Guiana, that borders Northern Brazil, raises the possibility of hemoplasma infection in native Brazilian monkeys (CONTAMIN *et al*., 1999; MICHEL *et al*., 2000; NEIMARK *et al*., 2002). Our research group is currently conducting a survey in Brazilian peccaries (*Tayassu pecari* and *T. tajacu*) since *M. suis* infection was described in the USA (HANNON *et al.*, 1985).

2.1.4 Laboratory Animals

Mycoplasma coccoides (formerly known as *Eperythrozoon coccoides*), which causes a mild hemolytic anemia in laboratory and wild mice, has been described in laboratory mice in Brazil (ANDRADE JR *et al*., 1986, 1989). No further work has been published to date, and epidemiological, clinical and molecular characterization of *M. coccoides* in Brazil remains to be determined in a variety of animals.

2.1.5 Public Health

Hemotrophic mycoplasmas infect a wide variety of mammals. There are also a few anecdotal reports of human infections (DUARTE *et al*., 1992). Recently, the first molecularly-documented case of a human hemoplasma infection was found in Brazil where a *Mycoplasma haemofelis*-like organism was detected in a HIV-positive patient co-infected with *Bartonella henselae* (SANTOS *et al.*, 2008b). Although this finding may represent an isolated case with no epidemiological consequence, it emphasizes the need for further research on the zoonotic potential of hemoplasma species.

2.2 CONCLUSION

Studies regarding to hemoplasmas are relatively common in Brazil. However they are not sufficient to determine whether hemoplasmas are important infectious agents in this country. Further studies should be conducted in order to better understand their clinical and epidemiologic impact on companion, wildlife, laboratory and production animals, as well as their role as zoonotic agents, particularly in immunocompromised patients.

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TABLE 1 - HEMOTROPHIC MYCOPLASMAS DESCRIBED IN BRAZIL

Cats	
	Mycoplasma haemofelis
	'*Candidatus* Mycoplasma turicensis'
	'*Candidatus* Mycoplasma haemominutum'
Dogs	
	Mycoplasma haemocanis
	'*Candidatus* Mycoplasma haematoparvum'
	Organism similar to '*Candidatus* Mycoplasma haemominutum'
	Organism similar to '*Candidatus* Mycoplasma turicensis'
Humans	
	Organism similar to *Mycoplasma haemofelis*
Pigs	
	Mycoplasma suis
	Organism similar to '*Candidatus* Mycoplasma haematoparvum'
Wild animals	
	Mycoplasma haemofelis
	'*Candidatus* Mycoplasma turicensis'
	'*Candidatus* Mycoplasma haemominutum'
Laboratory animals	
	Mycoplasma coccoides

3 DETECTION OF A NOVEL HEMOPLASMA BASED ON 16S rRNA GENE DNA IN CAPTIVE AND FREE-RANGING CAPYBARAS (*Hydrochaeris hydrochaeris*).

RESUMO

Duas species diferentes de hemoplasmas, *Mycoplasma coccoides* e *M. haemomuris*, são conhecidas por infectar pequenos roedores, como camundongos e ratos. Entretanto, não existem relatos de infecções por hemoplasmas em capivaras (*Hydrochaeris hydrochaeris*). O objetivo deste estudo foi determinar se esses hemoplasmas podem infectar capivaras do Sul do Brasil. Amostras de sangue de 31 animais: 10 capivaras de cativeiro e 21 de vida livre foram coletadas e o hematócrito e a proteína plasmática foram mensurados. Foi extraído o DNA das amostras e PCR para *M. coccoides* e *M. haemomuris* foram realizados. Usando o protocolo de PCR para *M. coccoides*, 64% das capivaras foram positivas, 80% de vida livre e 30% de cativeiro. A prevalência da infecção entre os grupos foi significativamente diferente (p = 0,001). O sequênciamento do gene 16S rRNA das amostras positivas sugerem que um novo isolado de hemoplasma foi identidade de 92% com o *M. coccoides* e 86% com o *M. haemomuris*. Todas as amostras das capivaras foram negativas para infecção por *M. haemomuris*. O DNA do gene constitutivo foi amplificado com sucesso em todas as amostras. Esta é a primeira evidência de uma infecção por hemoplasma em capivaras.

Palavras-chave: *Mycoplasma coccoides. Mycoplasma haemomuris.* Hemoplasma. Capivara (*Hydrochaeris hydrochaeris*). Roedores.

ABSTRACT

Two different species of hemoplasmas, *Mycoplasma coccoides* and *M. haemomuris*, are known to infect small rodents such as mice and rats. However, there are no previous reports of hemoplasma infection in capybara (*Hydrochaeris hydrochaeris*). The aim of our study was to determine whether these hemoplasmas might infect capybaras from Southern Brazil. Blood samples from 31 animals: 10 captive and 21 free-ranging capybaras were collected and Packed Cell Volume and Total Plasma Protein were measured. DNA was extracted and PCR assays for *M. coccoides* and *M. haemomuris* were performed. Using the *M. coccoides*-PCR assay 64% of the capybaras were positive, 80% free-ranging and 30% from captive animals. The prevalence of infection between the groups was significantly different (p = 0.001). Sequencing of the nearly entire 16S rRNA gene from the positive samples suggested a novel hemoplasma isolate with identity of 92% with *M. coccoides* and 86% with *M. haemomuris*. All capybara samples were negative for *M. haemomuris* infection. DNA of a housekeeping gene was successfully amplified from all samples. This is the first evidence of a hemoplasma infection in capybaras.

Key words: *Mycoplasma coccoides. Mycoplasma haemomuris.* Hemoplasma. Capybara (*Hydrochaeris hydrochaeris*). Rodent.

3.1 INTRODUCTION

Mycoplasma coccoides (*Eperythrozoon coccoides*) and *Mycoplasma haemomuris* (*Haemobartonella muris*) are blood parasites of wild and laboratory rodents (NEIMARK *et al*., 2001; NEIMARK *et al*., 2005; ZHANG; RIKIHISA, 2002). They are small, pleomorphic non-cultivable bacteria that lack a cell wall and attach to the surface of red blood cells (MESSICK, 2004). There are no previous reports of hemoplasma infection in capybaras (*Hydrochaeris hydrochaeris*). Capybaras are the biggest rodents in the world and live in forests and glasslands of Brazil and other South and Central American Countries (HENRIQUE-SILVA *et al*., 2007). The objective of our study was to determine whether hemoplasmas are found in captive and free-ranging capybaras in Southern Brazil using PCR-based assays.

3.2 MATERIALS AND METHODS

3.2.1 Blood collection

Blood samples were taken from 31 capybaras (10 captive and 21 free-ranging) at Bela Vista Sanctuary, Foz do Iguaçu, Parana State, Brazil under specific chemical restraint and stored at 4ºC for two hours until hematological analyses. Thereafter, samples were stored at -20ºC until molecular procedures were run. Animal and laboratory procedures were performed under regulations of the Brazilian Institute for the Environment and the Renewable Resources (IBAMA).

The packed cell volume (PCV) and total plasma protein (TPP) were measured by routine centrifugation and refractometry techniques; a PCV of 0·44 L/L or less and a TPP of 60 g/L or less were used as indicators of anemia and hypoproteinemia, respectively, in the capybaras (AROUCA *et al*., 2000).

3.2.2 DNA extraction

DNA was extracted from 200 µL blood using a commercially available kit according to the manufacturer's instructions (Illustra™ GFX™ Genomic Blood DNA Purification Kit, GE Healthcare, Buckinghamshire, UK).

3.2.3 GAPDH-PCR assay

A PCR for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed to ensure successful DNA extraction, as previously described (BIRKENHEUER *et al*., 2003).

3.2.4 *M. coccoides*-PCR assay

All samples were initially screened by PCR using a bacterial universal primer set (MESSICK *et al*., 1998). Positive samples were sequenced. A conventional PCR-assay for the detection of 16S rRNA gene of *M. coccoides* was developed. Specific primers for *M. coccoides* detection were manually designed and commercially synthesized by IDT® (Integrated DNA Technologies, Coralville, IA, USA). The primer set used to amplify a 305 bp was McoccF1 (5'-ATG ATG GTA CCT CCT GAA TA-3') and McoccR1 (5'-CAT TTG CTA CCC ACA CTT-3'). The predicted 305 bp products were separated by electrophoresis in a 1% agarose gel containing 5 µg/mL ethidium bromide and visualized under UV light. Specificity was evaluated using known positive samples for *M. haemofelis*, '*Candidatus* M. haemominutum', '*Candidatus* M. turicensis', *M. haemocanis*, *M. suis*, *M. wenyonii*, *M. haemomuris* and *Bartonella henselae*. To determine the limit of detection, 5 µL of plasmid control DNA was 10-fold serially diluted.

3.2.5 *M. haemomuris PCR assay*

A positive control for *M. haemomuris* was kindly provided by Dr. Yasuko Rikihisa, Ohio State University. The protocol used for amplification of the 16S rRNA gene of *M. haemomuris* was described by Zhang e Rikihisa (2002).

3.2.6 Sequence of 16S rRNA gene

Using universal primers, positive samples for *M. coccoides* from 1 captive and 3 free-ranging capybara, were amplified using Platinum® *Taq* High Fidelity DNA Polymerase. PCR products of 1,346 bp were purified from the agarose gel (Zymoclean™ Gel DNA Rec. Kit; Zymo Res. Corp., Orange, CA, USA) and sequenced (Purdue Genomics Core Facility at Purdue University, West Lafayette, IN, USA).

3.2.7 Phylogenetic analysis

The 16S rRNA gene sequences from the capybara isolates were aligned with sequences from GenBank database (Fig. 1) using Clustal W2 (EMBL-EBI). A phylogenetic tree was constructed using the software Mega 4.1 (KUMAR *et al*., 2008) with the neighbor-joining method from a distance matrix corrected for nucleotide substitutions by the Kimura two-parameter model (KIMURA, 1980). The data set was resampled 1,000 times to generate bootstrap values.

3.2.8 Nucleotide sequence accession numbers

The nucleotide sequences of two hemoplasmas isolates from capybaras were submitted to the GenBank database under the accession numbers FJ667773 and FJ667774.

3.2.9 Statistical analysis

A non-parametric Mann-Whitney test was used to compare the mean PCV and TPP concentrations. A descriptive analysis of the variable (positive/negative for hemoplasma infection) collected from each group was performed. The Chi-Square or Fisher's exact test was used for univariate analysis of selected variables. The results were considered significantly different when $p < 0.05$. Data were compiled and analyzed in Stata Statistical Software (StataCorp, Col. Station, TX, USA).

3.3 RESULTS

3.3.1 PCR -assay for *M. coccoides* detection

The initial concentration of the novel hemoplasma plasmid evaluated by continueous optical scan of UV absorbance from 220 to 320nm; the DNA was pure, having a concentration of 125.02 ηg/μL. Amplification of the hemoplasma plasmid DNA used as a positive control was efficient up to 10^{-7} dilution (0,0125pg/μL). The primer set did not amplify DNA from any of the other species tested.

3.3.2 Detection of hemoplasmas

For all capybara samples analyzed with the *M. coccoides* protocol, 20/31 (64%) were positive, including 17/21 (80%) free-ranging and 3/10 (30%) captive capybaras. The prevalence of hemoplasma detection was significantly greater for free-ranging than captive animals (p = 0.01). Using the *M. haemomuris* protocol all capybara samples were negative, however these samples consistently amplified the GAPDH gene.

3.3.3 PCV and TTP

PCV for captive (0.43 L/L) and free-ranging (0.41 L/L) capybaras was statistically different (p=0.005). The PCV for all *M. coccoides*–PCR positive capybaras (0.41 L/L) was significantly different when compared to *M. coccoides*–PCR negative capybaras (0.42 L/L) (p=0.027). In anemic capybaras, two captive (50%) and 14 free-ranging (79%) tested positive by PCR using the *M.coccoides* protocol.

The median and range of the TPP concentrations for captive and free-ranging capybaras was 76.0 (70.0 – 82.0) and 68.0 (60.0 – 104.0) g/L, respectively. These medians were significantly different (p = 0.008) with lower values for free-ranging capybaras. TPP for all *M. coccoides*–PCR positive capybaras, 68.0 g/L, was significantly lower when compared with the *M. coccoides*–PCR negative capybaras, 76.0 g/L (p = 0.027).

3.3.4 Phylogenetic analysis

Three of the 16S rRNA gene sequences of a hemoplasma isolate obtained from the blood of capybaras (one captive and two free-ranging) had 100% sequence identity to each other (isolate #1), however when aligned with an isolate from the blood of another free-ranging capybara, a 17 bp difference (98% identity) was consistently identified (isolate #2). Comparison between the 2 hemoplasma isolates of capybaras to those from GenBank database revealed the sequences had highest identity to *M. coccoides* (91.6% to 92%) and '*Candidatus M. turicensis*' (91% to 91.4%). Construction of a phylogenetic tree confirmed this close relationship (Fig. 1), however the capybara isolates branched away from *M. haemomuris* and were more divergence to other hemoplasma species.

3.4 DISCUSSION

This article is the first report of a novel hemoplasma in capybara and provides data regarding a novel species and its prevalence of infection in the population studied. Since hemoplasma species have not been successfully cultured, detection of new species must rely on indirect tools such as molecular DNA analysis. The 16S rRNA gene sequence from the novel hemoplasma of the capybara was more closely related to *M. coccoides*, but with only a 92% identity score. According to the cutoff value of 16S rRNA gene sequence identity for species definition, "*two bacterial isolates would belong to different species if the similarity in the 16S rRNA gene sequence between them were less than 97 %*" (DRANCOURT; RAOULT, 2005). Moreover, the 16S rRNA gene sequences have been widely used in environmental microbiology for assigning uncultivable organisms as new species (HANAGE *et al*., 2006).

Interestingly, a significantly higher prevalence of hemoplasma infections was found in free-ranging capybaras (80%) than in captive animals (30%). This data is

similar to that reported by Willi *et al*. (2007), which found 53% of the blood samples from free-living *Apodemus* mice in Switzerland were positive by PCR for *M. coccoides*. Thus, our data suggests the novel hemoplasma isolate from the capybara is a new species. Two isolates of this hemoplasma have been submitted to GenBank; the author's speculation is that these are 2 strains of the same species.

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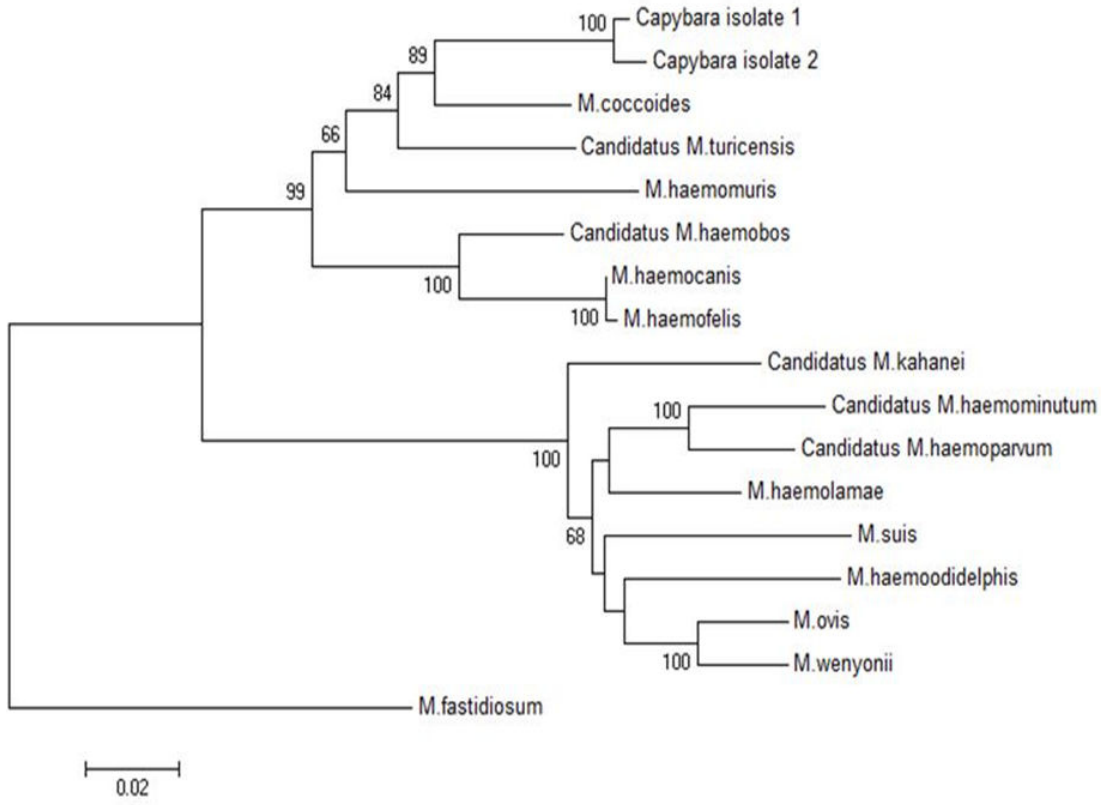
FIGURE 1 - PHYLOGENETIC TREE BASED ON 16S RRNA GENE SEQUENCES SHOWING THE RELATIONSHIP OF THE CAPYBARA HEMOPLASMA ISOLATES (FJ667773 AND FJ667774) TO THE OTHER HEMOPLASMA SPECIES USING A NEIGHBOR JOINING METHOD. *M. COCCOIDES* (AY171918), *M. HAEMOMURIS* (U82963), *M. HAEMOFELIS* (AY150985), '*CANDIDATUS* MYCOPLASMA HAEMOMINUTUM' (AY297712), '*CANDIDATUS* MYCOPLASMA TURICENSIS' (DQ825450), *M. HAEMOCANIS* (AY150973), '*CANDIDATUS* MYCOPLASMA HAEMOPARVUM' (AY532390), '*CANDIDATUS* MYCOPLASMA KAHANEI' (AF338269), *M. WENYONII* (AF016546), '*CANDIDATUS* M. HAEMOBOS' (EF460765), *M. SUIS* (AY492086), '*CANDIDATUS* MYCOPLASMA HAEMOLAMAE' (AF306346), '*CANDIDATUS* MYCOPLASMA HAEMODIDELPHIDIS' (AF178676), *M. OVIS* (AF338268) AND *M. FASTIDIOSUM* (NR_024987). *M. FASTIDIOSUM* WAS USED AS OUTGROUP. BOOTSTRAP PERCENTAGE VALUES ARE GIVEN AT THE NODES OF THE TREE; VALUES OF < 50 ARE NOT SHOWN. SCALE BAR, 0.02 UNIT.



4 CONCLUSÕES FINAIS

Estudos sobre hemoplasmas em diversas espécies animais no Brasil, em sua maioria, abrangem dados de prevalência em determinadas cidades do país. Pesquisas envolvendo a patogenia, modos de transmissão, manifestações clínicas e morbidade da doença são necessários, visto que algumas populações da bactéria diferem geneticamente das já descritas em outros países.

Apesar da infecção por hemoplasmas já ter sido descrita em cães, gatos, suínos, bovinos, felídeos selvagens e roedores, algumas espécies animais estão presente apenas em determinadas regiões do mundo, como no caso da capivara em nosso continente.

O presente estudo determinou a primeira descrição de infecção por hemoplasmas em capivaras, que apesar de ser um roedor, não se encontravam parasitadas pelas espécies *M. coccoides* e *M. haemomuris*, anteriormente descritas nos roedores. Nossos dados sugerem que o hemoplasma detectado seja uma nova espécie de *Mycoplasma* sp. baseado em análise filogenética do gene 16S rRNA da bactéria.

Visando a caracterização desta nova espécie, o próximo passo do estudo deverá envolver o achado em lâmina de esfregaço sanguíneo do *Mycoplasma* sp. parasitando os eritrócitos das capivaras. Após isso, imagens de microcopia eletrônica deverão ser capturadas a fim de se estabelecer o parasitismo extracelular.

Visto que este trabalho teve como objetivo apenas a detecção e comparação genética de hemoplasmas em capivaras, estudos aprofundados que envolvam avaliação de fatores de risco, como presença de carrapatos, pulgas e/ou piolhos, idade, sexo, associados a sinais clínicos da doença, bem como co-infecção com outros agentes infecciosos são necessários para estabelecer o mecanismo de ação e o potencial patogênico do *Mycoplasma* sp. encontrado.

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ANEXOS

ANEXO 1 – QUADROS DO PROTOCOLO DA PCR PARA DETECÇÃO DO *Mycoplasma coccoides*.

QUADRO 1 – PROTOCOLO DA REAÇÃO PARA DETECÇÃO DO GENE 16S rRNA DO *Mycoplasma coccoides* PELA REAÇÃO EM CADEIA DA POLIMERASE (PCR), PARA REAÇÃO FINAL DE 25 µL.

	[ ] Estoque	**[ ] Reação**	**µL**
H_2O	-	-	10,25
Tampão	5x	1x	5
$MgCl_2$	25 mM	1,5	1,5
dNTPs	2,5 mM	0,2	2
McoccF1*	10 pmol	0,2	0,5
McoccR1*	10 pmol	0,2	0,5
***Taq* DNA Polimerase**	5 U/L	1,25	0,25
DNA da amostra	-	-	5

***** "Primers" (iniciadores): McoccF (senso) e McoccR (antisenso)
Símbolos: [ ] – Concentração
Abreviações: H_2O – água; $MgCl_2$ – Cloreto de Magnésio; dNTPs - Desoxirribonucleotídeos Fosfatados; mM – Milimolar; pmol – picomole; µL – microlitro;

QUADRO 2 – CONDIÇÕES DA TERMOCICLAGEM PARA DETECÇÃO DO GENE 16S rRNA DO *Mycoplasma coccoides* PELA REAÇÃO EM CADEIA DA POLIMERASE (PCR).

Fases do Ciclo	**Temperatura / Tempo**
1 – Desnaturação Inicial	95ºC / 2 minutos
2 – Desnaturação	94ºC / 1 minuto
3 – Anelamento	53ºC / 30 segundos
4 – Extensão	72ºC / 30 segundos
5 – Ciclos	Volta para etapa 2, 34 vezes
6 – Extensão Final	72ºC / 5 minutos
7 – Refrigeração	4ºC / retirar

ANEXO 2 – TABELA COM VALORES MÉDIOS DE HEMATÓCRITO EM CAPIVARAS DE CATIVEIRO E VIDA LIVRE ASSOCIADOS A INFECÇÃO POR HEMOPLASMA

TABELA 1 – VALORES MÉDIOS E DESVIO PADRÃO DO HEMATÓCRITO, EM L/L, DE CAPIVARAS DE CATIVEIRO E VIDA LIVRE ASSOCIADOS A ANEMIA E INFECÇÃO POR HEMOPLASMA.

População (n)		PCR	
		+ (n) Ht (dp)	- (n) Ht (dp)
Vida Livre	Anêmicos (17)	(14) 0,37 (± 0,4)	(3) 0,38 (± 0,2)
	Não-Anêmicos (4)	(3) 0,44 (± 0,1)	(1) 0,45 (0)
Cativeiro	Anêmicos (5)	(2) 0,42 (0)	(3) 0,41 (± 0,2)
	Não-Anêmicos (5)	(1) 0,44 (0)	(4) 0,46 (± 0,1)

Ht – Hematócrito, dp – Desvio padrão, n – Número de animais.

ANEXO 3 – FOTOS DE ELETROFORESE EM GEL DE AGAROSE À 1% CORADO COM BROMETO DE ETÍDEO À 1,5%.

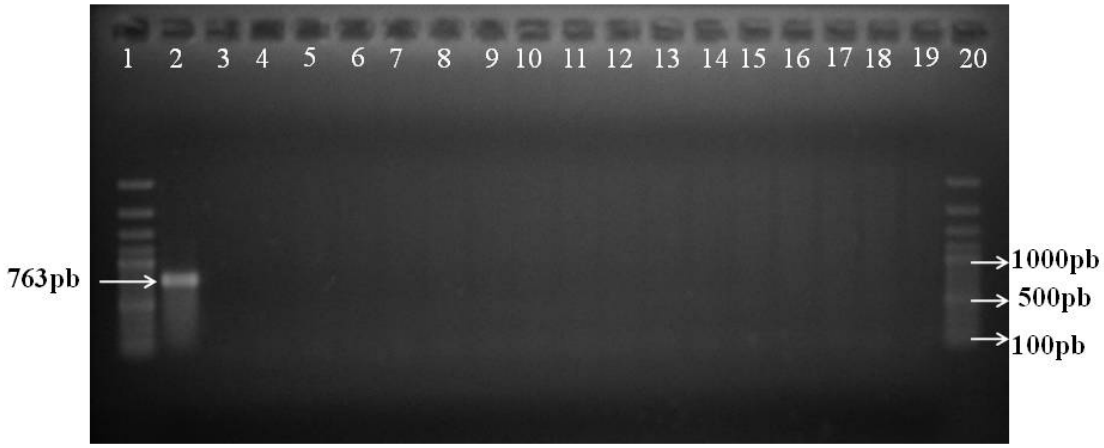

FIGURA 1 – PCR PARA DETECÇÃO DO GENE 16S RRNA DO *MYCOPLASMA HAEMOMURIS* EM CAPIVARAS (*HYDROCHAERIS HYDROCHAERIS*) UTILIZANDO OS PRIMERS HM16S-1F E HM16S-2R. COLUNA 1, MARCADOR DE PESO MOLECULAR 100PB; COLUNA 2, CONTROLE POSITIVE (DNA DO *M. HAEMOMURIS*); COLUNA 3, CONTROLE NEGATIVE (ÁGUA); COLUNAS 4 – 19, AMOSTRAS DE CAPIVARAS NEGATIVAS; COLUNA 20, MARCADOR DE PESO MOLECULAR 100PB.

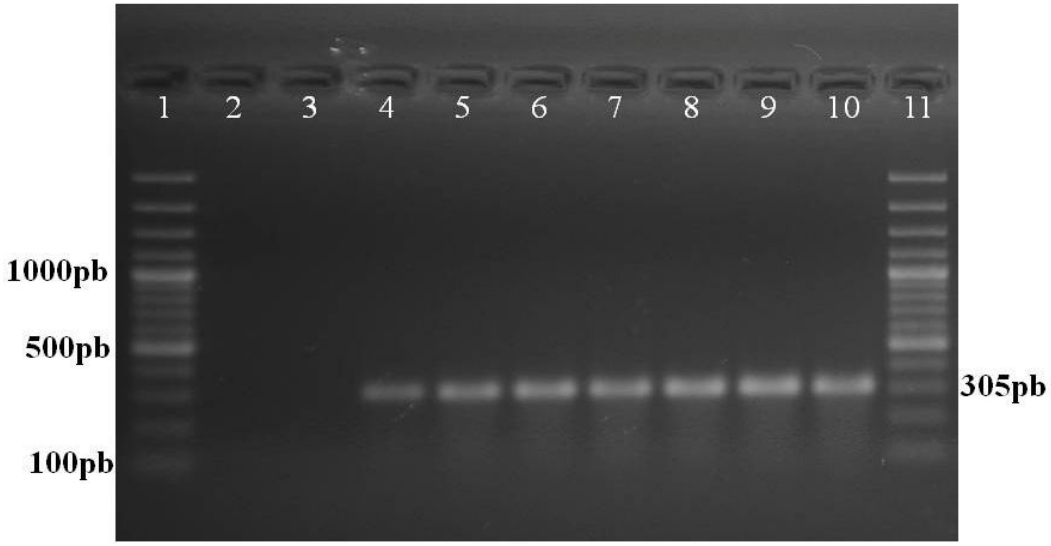

FIGURA 2 – EXPERIMENTO DE GRADIENTE DE CONCENTRAÇÃO DE CLORETO DE MAGNÉSIO (MGCL) PARA DETECÇÃO DO GENE 16S RRNA DO *MYCOPLASMA COCCOIDES* UTILIZANDO OS PRIMERS MCOCCF1 E MCOCCR1. COLUNA 1 E 11, MARCADOR DE PESO MOLECULAR 100PB; COLUNA 2, CONTROLE NEGATIVO (ÁGUA); COLUNAS 3 A 10, CORRESPONDEM AS RESPECTIVAS CONCENTRAÇÕES DE MGCL EM MM 0,5; 1,0; 1,5; 2,0; 2,5; 3,0; 3,5 E 4,0.

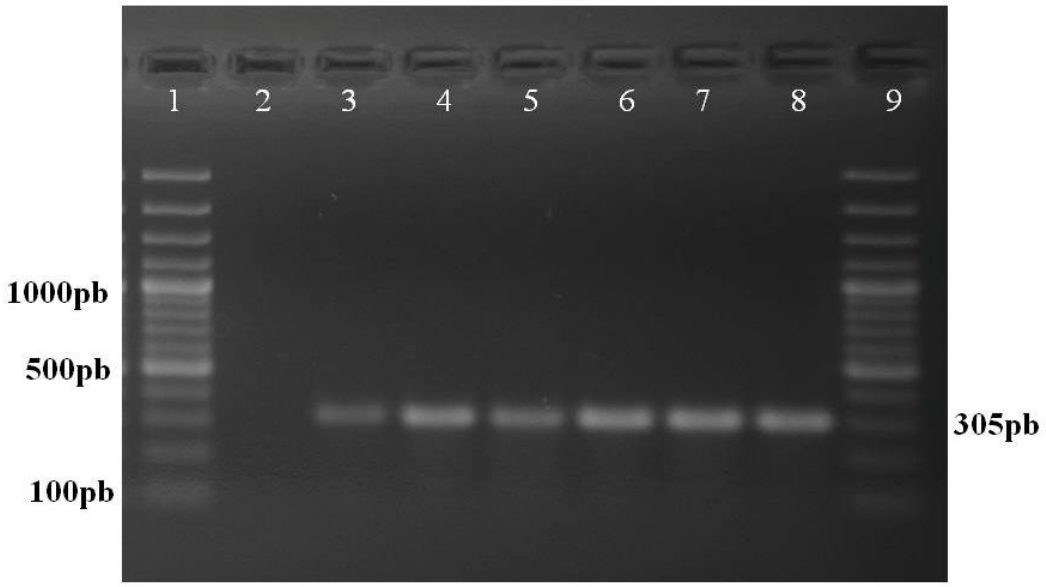

FIGURA 3 – EXPERIMENTO DE GRADIENTE DE CONCENTRAÇÃO DE PRIMERS PARA DETECÇÃO DO GENE 16S RRNA DO *MYCOPLASMA COCCOIDES* UTILIZANDO OS PRIMERS MCOCCF1 E MCOCCR1. COLUNA 1 E 9, MARCADOR DE PESO MOLECULAR 100PB; COLUNA 2, CONTROLE NEGATIVO (ÁGUA); COLUNAS 3 A 8, CORRESPONDEM AS RESPECTIVAS CONCENTRAÇÕES DE PRIMERS EM PMOL 0,25; 0,5; 0,75; 1,0; 1,25 E 1,5.

FIGURA 4 – PCR PARA DETECÇÃO DO GENE 16S RRNA DO *MYCOPLASMA SUIS* EM CATETOS (*TAYASSU TAJACU*) E QUEIXADAS (*TAYASSU PECARI*) UTILIZANDO OS PRIMERS ESUIS-F2 E ESUIS-R2. COLUNA 1, MARCADOR DE PESO MOLECULAR 100PB; COLUNA 2, CONTROLE POSITIVE (DNA DO *M. SUIS*); COLUNA 3, CONTROLE NEGATIVE (ÁGUA); COLUNAS 4 – 19, AMOSTRAS DE CATETOS NEGATIVAS; COLUNA 20, MARCADOR DE PESO MOLECULAR 100PB.

ANEXO 4 – IMAGENS DOS LOCAIS E PROCEDIMENTOS DA COLETA DE AMOSTRAS EM CAPIVARAS DE CATIVEIRO E VIDA LIVRE, NO REFÚGIO BIOLÓGICO BELA VISTA, ITAIPU BINACIONAL, FOZ DO IGUAÇU, PARANÁ, BRASIL.

FIGURA 1 – IMAGEM AÉREA DA ITAIPU BINACIONAL. VL – LOCAL DE COLETA DE AMOSTRAS DAS CAPIVARAS DE VIDA LIVRE, C – RECINTO DAS CAPIVARAS DE CATIVEIRO.

FIGURA 2 – CONTEÇÃO EM CAPIVARA DE VIDA LIVRE UTILIZANDO PUÇÁ.

FIGURA 3 – COLETA DE SANGUE DA VEIA FEMORAL EM CAPIVARA DE VIDA LIVRE.

ANEXO 5 – SEQUÊNCIA PARCIAL DE NUCLEOTÍDEOS DO GENE 16S rRNA DO CLONE 1 DE *Mycoplasma* sp. NÃO-CULTIVÁVEL, ARMAZENADA NO GENBANK. NÚMERO DE ACESSO FJ667773.

>gi|224551590|gb|FJ667773.1| Uncultured Mycoplasma sp. clone 1 16S ribosomal RNA gene, partial sequence

ATGCAAGTCGAACGGACTTTGCTTCGGCAAAGTTAGTGGCGAACGGGTGAGTAATGCATATTTAACATAC
CCCTAGGAGGGGAATAGCCACCTGAAAAGGTGATTAATACCCCATAGTAGCTCCCTCGCATGAGGTAGCT
TTAAAGGTTTACTCCGCCTAGGGATTGGAATATGTTCTACTAGTTTGTTGGTGAGGTAAAGGCTCACCAA
GACGATGATAGATAGCTGGTCTTAGAGGATGAACAGCCACAATGGGATTGAGATACGGCCCATATTCTTA
CGGGAAGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAATGCCATGTGAACGATGA
AGGCCTATTTGGTCGTAAAGTTCTTTTAGAGGGGAAGACTTTGACGGTACCCTCTGAATAAGTGACAGCA
AACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCGAGCGTTATTCGGTTTTATTGGGCGTAAAGCAA
GCGCAGGCGGATGAACAAGTTCTGTGTTAAAAGCAGCTGCTCAACAGTTGTTTGCACCGAATACTGTTCG
TCTAGAATGTGGTAGGAAGTTTTGGAATTAAATATGGAGCGGTGGAATGTGTAGATATATTTAAGAACAC
CAGAGGCGAAGGCGAAAACTTAGGCCATCATTGACGCTTAGGCTTGAAAGTGTGGGTAGCAAATGGGATT
AGATACCCCAGTAGTCCACACCGTAAACGATGGGTATTGGATGTCGGGCTTTGTGGCTCGGTGTTGTAGC
TTACGTGTTAAATACCCCGCCTGGGTAGTATATATGCAAATATGAAACTCAAAGGAATTGACGGGGACCT
GAACAAGTGGTGGAACATGTTGCTTAATTCGATAATACACGAAAAACCTTACCAGGGTTTGACATCCCTT
GCGAAACCGTGGAAACACGGCGGAGGTTATCAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
ATGAGATGTTTGGTTAAGTCCCGCAACGAGCGCAACCCTACCCCTTAGTTGTTTGTCTAAGGAGACTGCA
CAGTAATGTAGAGGAAGGATGGGATCACGTCAAATCATCATGCCCCTTATGCCCTGGGCTGCAAACGTGT
TACAATGGTAGATACAATATGTCTGCAAACCAGCGATGGTAAGCTAATCATCAAAATCTATCTCAGTCCG
GATAAAAGGCTGCAATTCGCCTTTTTGAAGTTGGAATCACTAGTAATCCCGTGTCAGCTATATCGGGGTG
AATACGTTCCCAGGTCTTGTACACACCGCCCGTCAAACTATGAGAGGAAGGGGCGTTTAAAAATACATTT
ATTTGTATCTAGAACGA

ANEXO 6 – SEQUÊNCIA PARCIAL DE NUCLEOTÍDEOS DO GENE 16S RRNA DO CLONE 2 DE *Mycoplasma* sp. NÃO-CULTIVÁVEL, ARMAZENADA NO GENBANK. NÚMERO DE ACESSO FJ667774.

>gi|224551591|gb|FJ667774.1| Uncultured Mycoplasma sp. clone 2 16S ribosomal RNA gene, partial sequence

ATGCAAGTCGAACGGACTTTTCCTTGGAAAAGTTAGTGGCGAACGGGTGAGTAATGCATATTTAACATAC
CCCTAGGAGGGGCATAGCCGCCTGAAAAGGCGATTAATACCCCATAGTAGCTCCCTCGCATGAGGTAGCT
TTAAAGGTTTACTCCGCCTAGGGATTGGAATATGTTCTACTAGTTTGTTGGTGAGGTAAAGGCTCACCAA
GACGATGATAGATAGCTGGTCTTAGAGGATGAACAGCCACAATGGGATTGAGATACGGCCCATATTCTTA
CGGGAAGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAATGCCATGTGAACGATGA
AGGCCTATTTGGTCGTAAAGTTCTTTTAGGAGGGAAGACTTTGACGGTACCTCCTGAATAAGTGACAGCA
AACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCGAGCGTTATTCGGTTTTATTGGGCGTAAAGCAA
GCGCAGGCGGATGAACAAGTTCTGTGTTAAAAGCAGCTGCTCAACAGTTGTTTGCACCGAATACTGTTCG
TCTAGAATGTGGTAGGAAGTTTTGGAATTAAATATGGAGCGGTGGAATGTGTAGATATATTTAAGAACAC
CAGAGGCGAAGGCGAAAACTTAGGCCATCATTGACGCTTAGGCTTGAAAGTGTGGGTAGCAAATGGGATT
AGATACCCCAGTAGTCCACACCGTAAACGATGGGTATTGGATGTCGGGCTTTGCGGCTCGGTGTTGTAGC
TTACGTGTTAAATACCCCGCCTGGGTAGTATATATGCAAATATGAAACTCAAAGGAATTGACGGGGACCT
GAACAAGTGGTGGAACATGTTGCTTAATTCGATAATACACGAAAAACCTTACCAGGGTTTGACATCCCTT
GCGAAACCGTGGAAACACGGCGGAGGTTATCAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
ATGAGATGTTTGGTTAAGTCCCGCAACGAGCGCAACCCTACCCCTTAGTTGTTTTTCTAAGGAGACTGCA
CAGTAATGTAGAGGAAGGATGGGATCACGTCAAATCATCATGCCCCTTATGCCCTGGGCTGCAAACGTGT
TACAATGGTGGATACAATATGTCTGCAAACCAGCGATGGTAAGCTAATCATTAAAATTCATCTCAGTCCG
GATAAAAGGCTGCAATTCGCCTTTTTGAAGTTGGAATCACTAGTAATCCCGTGTCAGCTATATCGGGGTG
AATGCGTTCCCAGGTCTTGTACACACCGCCCGTCAAACTATGAGAGGAAGGGGCGTTTAAAAATACATTT
ATTTGTATCTAGAACGA

ANEXO 7 – ALINHAMENTO DAS SEQUÊNCIAS PARCIAIS DE NUCLEOTÍDEOS DO GENE 16S RRNA DOS CLONES 1 E 2 DE *Mycoplasma* SP. NÃO-CULTIVÁVEL, ARMAZENADAS NO GENBANK, UTILIZANDO O PROGRAMA CLUSTAL W2. NÚMEROS DE ACESSO FJ667773 E FJ667774, EVIDENCIANDO OS 18 PARES DE BASE (pb) DIFERENTES.

```
Clone 1         ATGCAAGTCGAACGGACTTTGCTTCGGCAAAGTTAGTGGCGAACGGGTGAGTAATGCATA 60
Clone 2         ATGCAAGTCGAACGGACTTTTCCTTGGAAAAGTTAGTGGCGAACGGGTGAGTAATGCATA 60
                ******************* * * ** ********************************

Clone 1         TTTAACATACCCCTAGGAGGGGAATAGCCACCTGAAAAGGTGATTAATACCCCATAGTAG 120
Clone 2         TTTAACATACCCCTAGGAGGGGCATAGCCGCCTGAAAAGGCGATTAATACCCCATAGTAG 120
                ********************** ****** ********** ******************

Clone 1         CTCCCTCGCATGAGGTAGCTTTAAAGGTTTACTCCGCCTAGGGATTGGAATATGTTCTAC 180
Clone 2         CTCCCTCGCATGAGGTAGCTTTAAAGGTTTACTCCGCCTAGGGATTGGAATATGTTCTAC 180
                ************************************************************

Clone 1         TAGTTTGTTGGTGAGGTAAAGGCTCACCAAGACGATGATAGATAGCTGGTCTTAGAGGAT 240
Clone 2         TAGTTTGTTGGTGAGGTAAAGGCTCACCAAGACGATGATAGATAGCTGGTCTTAGAGGAT 240
                ************************************************************

Clone 1         GAACAGCCACAATGGGATTGAGATACGGCCCATATTCTTACGGGAAGCAGCAGTAGGGAA 300
Clone 2         GAACAGCCACAATGGGATTGAGATACGGCCCATATTCTTACGGGAAGCAGCAGTAGGGAA 300
                ************************************************************

Clone 1         TCTTCCACAATGGACGAAAGTCTGATGGAGCAATGCCATGTGAACGATGAAGGCCTATTT 360
Clone 2         TCTTCCACAATGGACGAAAGTCTGATGGAGCAATGCCATGTGAACGATGAAGGCCTATTT 360
                ************************************************************

Clone 1         GGTCGTAAAGTTCTTTTAGAGGGGAAGACTTTGACGGTACCCTCTGAATAAGTGACAGCA 420
Clone 2         GGTCGTAAAGTTCTTTTAGGAGGGAAGACTTTGACGGTACCTCCTGAATAAGTGACAGCA 420
                ******************  ******************  ******************

Clone 1         AACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCGAGCGTTATTCGGTTTTATTGGG 480
Clone 2         AACTATGTGCCAGCAGCTGCGGTAATACATAGGTCGCGAGCGTTATTCGGTTTTATTGGG 480
                ************************************************************

Clone 1         CGTAAAGCAAGCGCAGGCGGATGAACAAGTTCTGTGTTAAAAGCAGCTGCTCAACAGTTG 540
Clone 2         CGTAAAGCAAGCGCAGGCGGATGAACAAGTTCTGTGTTAAAAGCAGCTGCTCAACAGTTG 540
                ************************************************************

Clone 1         TTTGCACCGAATACTGTTCGTCTAGAATGTGGTAGGAAGTTTTGGAATTAAATATGGAGC 600
Clone 2         TTTGCACCGAATACTGTTCGTCTAGAATGTGGTAGGAAGTTTTGGAATTAAATATGGAGC 600
                ************************************************************

Clone 1         GGTGGAATGTGTAGATATATTTAAGAACACCAGAGGCGAAGGCGAAAACTTAGGCCATCA 660
Clone 2         GGTGGAATGTGTAGATATATTTAAGAACACCAGAGGCGAAGGCGAAAACTTAGGCCATCA 660
                ************************************************************

Clone 1         TTGACGCTTAGGCTTGAAAGTGTGGGTAGCAAATGGGATTAGATACCCCAGTAGTCCACA 720
Clone 2         TTGACGCTTAGGCTTGAAAGTGTGGGTAGCAAATGGGATTAGATACCCCAGTAGTCCACA 720
                ************************************************************
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Clone 1         CCGTAAACGATGGGTATTGGATGTCGGGCTTTGTGGCTCGGTGTTGTAGCTTACGTGTTA 780
Clone 2         CCGTAAACGATGGGTATTGGATGTCGGGCTTTGCGGCTCGGTGTTGTAGCTTACGTGTTA 780
                ******************************** ************************

Clone 1         AATACCCCGCCTGGGTAGTATATATGCAAATATGAAACTCAAAGGAATTGACGGGGACCT 840
Clone 2         AATACCCCGCCTGGGTAGTATATATGCAAATATGAAACTCAAAGGAATTGACGGGGACCT 840
                ************************************************************

Clone 1         GAACAAGTGGTGGAACATGTTGCTTAATTCGATAATACACGAAAAACCTTACCAGGGTTT 900
Clone 2         GAACAAGTGGTGGAACATGTTGCTTAATTCGATAATACACGAAAAACCTTACCAGGGTTT 900
                ************************************************************

Clone 1         GACATCCCTTGCGAAACCGTGGAAACACGGCGGAGGTTATCAAGGTGACAGGTGGTGCAT 960
Clone 2         GACATCCCTTGCGAAACCGTGGAAACACGGCGGAGGTTATCAAGGTGACAGGTGGTGCAT 960
                ************************************************************

Clone 1         GGTTGTCGTCAGCTCGTGTCATGAGATGTTTGGTTAAGTCCCGCAACGAGCGCAACCCTA 1020
Clone 2         GGTTGTCGTCAGCTCGTGTCATGAGATGTTTGGTTAAGTCCCGCAACGAGCGCAACCCTA 1020
                ************************************************************

Clone 1         CCCCTTAGTTGTTTGTCTAAGGAGACTGCACAGTAATGTAGAGGAAGGATGGGATCACGT 1080
Clone 2         CCCCTTAGTTGTTTTCTAAGGAGACTGCACAGTAATGTAGAGGAAGGATGGGATCACGT 1080
                ************* ********************************************

Clone 1         CAAATCATCATGCCCCTTATGCCCTGGGCTGCAAACGTGTTACAATGGTAGATACAATAT 1140
Clone 2         CAAATCATCATGCCCCTTATGCCCTGGGCTGCAAACGTGTTACAATGGTGGATACAATAT 1140
                ************************************************ **********

Clone 1         GTCTGCAAACCAGCGATGGTAAGCTAATCATCAAAATCTATCTCAGTCCGGATAAAAGGC 1200
Clone 2         GTCTGCAAACCAGCGATGGTAAGCTAATCATTAAAATTCATCTCAGTCCGGATAAAAGGC 1200
                ****************************** *****  ********************

Clone 1         TGCAATTCGCCTTTTTGAAGTTGGAATCACTAGTAATCCCGTGTCAGCTATATCGGGGTG 1260
Clone 2         TGCAATTCGCCTTTTTGAAGTTGGAATCACTAGTAATCCCGTGTCAGCTATATCGGGGTG 1260
                ************************************************************

Clone 1         AATACGTTCCCAGGTCTTGTACACACCGCCCGTCAAACTATGAGAGGAAGGGGCGTTTAA 1320
Clone 2         AATGCGTTCCCAGGTCTTGTACACACCGCCCGTCAAACTATGAGAGGAAGGGGCGTTTAA 1320
                *** ********************************************************

Clone 1         AAATACATTTATTTGTATCTAGAACGA 1347
Clone 2         AAATACATTTATTTGTATCTAGAACGA 1347
                ***************************
```

ANEXO 8 – RESUMO PUBLICADO NOS ANAIS DO XI CONGRESSO E XVII ENCONTRO ASSOCIAÇÃO BRASILEIRA DE VETERINÁRIOS DE ANIMIAS SELVAGENS – ABRAVAS, 2008.

Bonat M., Sousa R., Monego F., Javorouski M., Lacerda O., Nakatani I.N., Vieira R.F.C., Molento M.B., Biesdorf S.M., Biondo A.W. **Detection of *Mycobacterium tuberculosis* in Real Time PCR in a captive collared peccary (*Tayassu tajacu*).** Pesquisa Veterinária Brasileira 28 (Supl.):1-2. Departamento de Medicina Veterinária, Universidade Federal do Paraná; Rua dos Funcionários 1540, Curitiba, PR 80035-050, Brazil. E-mail: abiondo@illinois.edu

Introduction: Tuberculosis is a chronic infection caused by isolates of the *Mycobacterium tuberculosis* complex, occurring in animal and human populations (Huard *et al*. 2003, Pate *et al*. 2006). The members of the complex, *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum* and *M. microti*, show a high degree of homogeneity in the nucleotide sequence within the group, although there is variance in certain aspects as pathogenicity and hosts (Huard *et al*. 2003). Recently, three new species have been introduced in the complex: *M. canettii* (Van Soolingen *et al*. 1997), *M. caprae* (Aranaz *et al*. 1999) and *M. pinnipedii* (Cousins *et al*. 2003). *Mycobacterium tuberculosis* species, demonstrated primarily as a human pathogen, have been more frequently related in wild species that live in close contact with human beings (Michalak *et al*. 1998) or domestic animals. Since Zoological Parks share susceptible populations of animals and humans, they have great importance in public health, particularly for Zoo workers, but also for visitors, which may be in direct contact (Oh *et al*. 2002). Moreover, close contact in captivity, the same areas for feeding and water supply, inadequate disinfection and the exposition to the visitors may contribute for tuberculosis maintenance in such places (Montali *et al*. 2001). The domestic and wild pigs are from the order *Artiodactyla*, families *Suidae* e *Tayassuidae*, respectively. Among the tayassuids, the caititus or collared peccaries, *Tayassu tajacu*, and white-lipped peccary, *Tayassu pecari*, are present in Brazil (Furtado & Kashivakura 2007, Margarido & Mangini 2001). The collared peccaries are found in a wide variety of habitats, from

the desert vegetation to the arid and tropical rain forest (Oliveira *et al*. 2004). They are extremely rustic, highly environment adapted and are also appreciated for their meat and skin. The potential to explore these animals in captivity may reduce predatory and illegal hunt (Furtado & Kashivakura 2007). Currently, collared peccary has been commercially exploited in Brazil for the supply of restaurants and supermarket offering the consumption of exotic meat. This demand has increased recently due to rising concerns of the population with healthier food habits (Costa *et al*. 2007). Due to the large number of commercial breeding collared peccaries are not included in the Official List of Brazilian Institute of Environment and Renewable Resources (IBAMA) as endangered species of Brazilian fauna (IBAMA, 2008). Although, it is listed on Appendix II in the Convention and International Trade in Endangered Species of Wild Fauna and Flora (CITES), the number of free-range animals is still unknown, as well as its sanitary status (CITES 2008, IBAMA 2008). In the present study we describe the detection and identification of *M. tuberculosis* in a collared peccary from the Curitiba Zoo, associating *post-mortem* diagnostic techniques for tuberculosis, including bacterial culture and molecular detection.

Materials and Methods: In July 2008 a male *T. tajacu* from the Curitiba Zoo, Paraná State, Southern Brazil, died presenting weight loss. Postmortem examination revealed diffusely mottled and non-collapsed lungs with multifocal to coalescing nodules. These nodules were white to yellowish and measured 0.1 to 0.4 cm in diameter, being observed on both pleural and cut surfaces. Additionally, mediastinic lymph nodes were enlarged with multiple white nodules (ranging from 0.1 to 0.5cm in diameter) on the cut surface involving cortical and medullar regions. Histologically severe, multifocal to coalescing granulomatous pneumonia and granulomatous lymphadenitis were present on hematoxylin and eosin (HE) sections and large amounts of acid-fast organisms were evidenced with a modified acid-fast stain (Fite-Faraco). Granulomas of lung collected from necropsy were sent to bacterial culture and Real-Time Polymerase Chain Reaction (real-time PCR). Samples were homogenized and decontaminated with NaOH 4% followed

by neutralization with HCl 1.0 N (Quinn *et al*. 2005). After neutralization, sediment was cultured on Lowenstein-Jensen solid medium and cultures were incubated at 37°C. *Mycobacterium sp*. colonies were observed from culture. The DNA extraction from sediment was performed using a standard method (Sambrook & Russel 2001). The real-time PCR confirmed *M. tuberculosis.* Real time PCR was performed according to (Takahashi & Nakayama, 2006) and modified by our group. A Fam probe was used and a quencher conjugated to a minor groove binder (MGB).

Discussion and Conclusion: To the author's knowledge, this is the first molecular identification of tuberculosis caused for *M. tuberculosis* in *T. tajacu*. In 2006 two collared peccaries, a female and a male died presenting severe respiratory signs. The necropsy showing purulent material into a hard mass in the lungs suggestive of tuberculosis infection, but no laboratory diagnosis was performed to confirm the disease. A case of infection by *M. tuberculosis* in a male tapir (*Tapirus terrestris*) have been described in 2006 in the same Zoo. Thus, the contact between infected captive wild animals may trigger the spread of disease in zoo populations. The hypothesis regarding the source of collared peccaries infection by *M. tuberculosis* could be an infected animal that arrived in zoo and remained in the same place with other healthy animals. Moreover, is possible that a former infected employee or zoo visitors could be the initial source of disease. Thus, the elaboration of a protocol for tuberculosis diagnosis in wild animals and tuberculosis control programmes concerning humans, domestic and wild animals is essential to minimize the risk of this infectious disease.

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Key-Words: Mycobacterium tuberculosis, tuberculosis, PCR, collared peccaries, Tayassu tajacu.

ANEXO 9 – ARTIGO A SER SUMETIDO A REVISTA BRASILEIRA DE PARASITOLOGIA VETERINÁRIA.

"Nota de Pesquisa"

Use of a *Mycoplasma suis*-PCR protocol for screening in a Tayassuids (*Tayassu tajacu* and *Tayassu pecari*) population

Uso de um protocolo de PCR para *Mycoplasma suis* para avaliação em uma população de Taiassuídeos (*Tayassu tajacu* and *Tayassu pecari*)

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Abstract

Mycoplasma suis is a hemotrophic bacteria of red blood cells of pigs and is the causative agente of eperythrozoonosis in swine. The diagnostic is made by direct examination of blood smears and PCR methods. The aim of the study was to screen the wild pigs caititus (*Tayassu tajacu*) and collared peccaries (*T. pecari*) for the infection by *M. suis* using stained blood smears and a PCR assay based on 16S rRNA gene. For this, 28 blood samples from caititus and collared peccaries from Southern Brazil were collected. DNA was extracted and PCR assay was performed. All samples were negatives in both methods. DNA of a housekeeping gene was successfully amplified from all samples. Screening studies are important to better indicate and guarantee the health status from an animal population.

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Key words: Eperythrozoonosis, caititus (*Tayassu tajacu*), collared peccaries (*Tayassu pecari*), wild pigs.

Resumo

O Mycoplasma suis é uma bactéria hemotrófica dos eritrócitos de porcos e é o agente causador da eperitrozoonose dos suínos. O diagnóstico é feito pelo exame direto de esfregaços sanguíneos e métodos de PCR. O objetivo do estudo foi avaliar porcos selvagens, catetos (*Tayassu tajacu*) e queixadas (*T. pecari*) para a infecção por *M. suis* utilizando esfregaços sanguíneos corados e teste de PCR baseado no gene 16S rRNA desta bactéria. Para isto, 28 amostras de sangue de catetos e queixadas do sul do Brasil foram coletadas. O DNA foi extraído e a PCR foi realizada. Todas as amostras foram negativas em ambos os métodos. O DNA do gene constitutivo GAPDH foi amplificado em todas as amostras. Estudos de avaliação são importantes para melhor indicar e garantir o estado sanitário de uma população animal.

Palavras-chave: Eperitrozoonose, cateto (*Tayassu tajacu*), queixada (*Tayassu pecari*), porcos selvagens.

Mycoplasma suis (*Eperythrozoon suis*) is the causative agent of eperythrozoonosis in swine, is an extracellular, rod-shaped, small and pleomorphic non-cultivated hemotrophic bacteria that attaches to the red blood cells of pigs (MESSICK, 2004; OLIVEIRA *et al*., 2004). The clinical signs of *M. suis* infection are variable and generally the animals present anorexia, weakness, depression, anemia, icterus and fever. The signs occur mainly during periods of stress, such as, weaning (HENRY, 1979; HEINRITZI, 1999).

The diagnosis of *M. suis* infection in pigs is based on microscopic observation of the parasites attached to the red blood cells in a Wright-Giemsa-stained blood smear; however this technique is not sensitive for the definitive diagnosis of infection, especially the chronic disease. Polymerase Chain Reaction

(PCR) based in the amplification of the 16S rRNA gene of *M. suis* has presented a highly specific and sensitive procedure that can be used to better detect chronic infections in pigs (MESSICK, 2004).

Mycoplasma suis is frequently detected in domestic pigs around the world, including Brazil (GUIMARÃES *et al*., 2007; HOELZLE *et al*., 2007). The domestic and wild pigs are present in the order *Artiodactyla*, in the families *Suidae* e *Tayassuidae*, respectively. Among the tayassuids, the caititus (*Tayassu tajacu*) and collared peccaries (*T. pecari*) are present in Brazil (FURTADO; KASHIVAKURA, 2007; MARGARIDO; MANGINI, 2001). To the author's knowledge, the only report of *Eperythrozoon* spp in *T. pecari* was made in Texas and based on the observation of inclusion-like attached to the red blood cells, by direct examination of blood smears stained with diff quick (HANNON *et al*., 1985).

The caititus is found in a wide variety of habitats, from the desert vegetation to the arid and tropical rain forest (OLIVEIRA *et al*., 2004), while the collared peccaries has a more restrict, fragmented and discontinuous geographic distribution, occurring from Southern Mexico to Northern Argentina. Those are extremely rustic and highly environment adapted animals. They are appreciated for their meat and skin with potential to be explored by captive breeding, reducing the predatory and illegal hunt (FURTADO; KASHIVAKURA, 2007).

Due to the large number of commercial breeding, caititus and collared peccaries are not included in the Official List of Brazilian Institute of Environment and Renewable Resources (IBAMA) as endangered species of Brazilian fauna (IBAMA, 2008). However, it is listed on Appendix II in the Convention and International Trade in Endangered Species of Wild Fauna and Flora (CITES); the number of free-range animals is still unknown, as its sanitary status.

Thus, the aim of the study was to screening tayassuids for the infection by *M. suis* using blood smears examination and a PCR assay based on 16S rRNA gene from this bacteria.

Blood samples were taken from 6 captive caititus at Bela Vista Sanctuary, Foz do Iguaçu, Parana State, Brazil and 6 collared peccaries and 16 caititus at the

Curitiba Zoo, Paraná State, Southern Brazil under specific chemical restraint and stored at 4ºC for two hours until hematological analyses. Thereafter, samples were stored at -20ºC until molecular procedures were run. Animal and laboratory procedures were performed under regulations of the Brazilian Institute for the Environment and the Renewable Resources (IBAMA).

Blood smears were prepared and stained with Diff-Quik. Examination was done using light microscopy at high magnification (40x and 100x objective lens). DNA was extracted from 200 µL blood using a commercially available kit according to the manufacturer's instructions (Ilustra^{TM} GFX^{TM} Genomic Blood DNA Purification Kit, GE Healthcare, Buckinghamshire, UK). A PCR for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed to ensure successful DNA extraction, as previously described (BIRKENHEUER *et al*., 2003). A PCR for the detection of the 16S rRNA gene of the *M. suis* was performed as previously described (GUIMARÃES *et al*., 2007). All reactions were performed using DNA from an infected domestic pig and DNA extracted from a non-infected pig as a positive and negative control, respectively.

Direct examinations of the blood smears from all animals were considered negative for *Mycoplasma*-like infection. The 28 samples from tayassuids were considered negative by the *M. suis*-PCR assay used. DNA of a housekeeping gene was successfully amplified from all samples.

Negative results observed in both methods did not ensure that animals are not infected by *Mycoplasma* spp. As blood smears is not a sensitive method and PCR-assay used was develop for the detection of the bacteria in domestic pigs, the tayassuids may be infected by a new *Mycoplasma* species, which cannot be amplified by the primer set used. Our results were different from Hannon *et al*. (1985), which found 71% of the blood samples from wild pigs in Texas were positive by blood smears examination for *Eperythrozoon* sp., but PCR was not performed.

Since the commercial production of caititus is growing steadily, screening studies are important to better indicate and guarantee the health status from an

animal population and, thus, avoid spreading of diseases and consequently economic losses.

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