UFRRJ

VETERINARY INSTITUTE

GRADUATE PROGRAM IN VETERINARY SCIENCES

THESIS

Diversity of the *Anaplasma marginale msp*1α gene and isolation and propagation of a novel genotype of *Anaplasma* sp. in IDE8 cells

Priscilla Nunes dos Santos

2020

FEDERAL RURAL UNIVERSITY OF RIO DE JANEIRO
VETERINARY INSTITUTE
GRADUATE PROGRAM IN VETERINARY SCIENCES

DIVERSITY OF THE *Anaplasma marginale msp*1α GENE AND ISOLATION AND PROPAGATION OF A NOVEL GENOTYPE OF *Anaplasma* sp. IN IDE8 CELLS

Priscilla Nunes dos Santos

Under supervision of the professor

Dr. Adivaldo Henrique da Fonseca

Under co-supervision of the professors

Drª. Cláudia Bezerra da Silva

Dr. Jenevaldo Barbosa da Silva

Thesis submitted as a partial requirement for obtaining a Science PhD degree in the Graduate Program in Veterinary Sciences.

Seropédica, RJ
June, 2020

UNIVERSIDADE FEDERAL RURAL DO RIO DE JANEIRO
INSTITUTO DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

DIVERSIDADE DO GENE *msp*1α DE *Anaplasma marginale* E ISOLAMENTO E PROPAGAÇÃO DE UM NOVO GENÓTIPO DE *Anaplasma* sp. EM CÉLULAS IDE8

Priscilla Nunes dos Santos

Sob a Orientação do Professor
Dr, Adivaldo Henrique da Fonseca

Sob a co-orientação dos Professores
Drª Cláudia Bezerra da Silva

Dr. Jenevaldo Barbosa da Silva

Tese submetida como requisito parcial para obtenção do grau de Doutora em Ciências no Programa de Pós-graduação em Ciências Veterinárias

Seropédica, RJ
Junho de 2020

Universidade Federal Rural do Rio de Janeiro
Biblioteca Central / Seção de Processamento Técnico

Ficha catalográfica elaborada
com os dados fornecidos pelo(a) autor(a)

S237d

Santos, Priscilla Nunes dos, 1990-
Diversity of the Anaplasma marginale msp1? gene and isolation and propagation of a novel genotype of Anaplasma sp. in IDE8 cells / Priscilla Nunes dos Santos. - Rio de Janeiro, 2020.
106 f.

Orientador: Adivaldo Henrique da Fonseca.
Coorientador: Jenevaldo Barbosa da Silva.
Coorientadora: Cláudia Bezerra da Silva.
Tese(Doutorado). -- Universidade Federal Rural do Rio de Janeiro, Curso de Pós Graduação em Ciências Veterinárias, 2020.

1. Diversity. 2. Rhipicephalus microplus. 3. IDE8. 4. msp1?. I. Fonseca, Adivaldo Henrique da, 1953-, orient. II. Silva, Jenevaldo Barbosa da, 1984-, coorient. III. Silva, Cláudia Bezerra da, 1984-, coorient. IV Universidade Federal Rural do Rio de Janeiro. Curso de Pós Graduação em Ciências Veterinárias. V. Título.

MINISTÉRIO DA EDUCAÇÃO
UNIVERSIDADE FEDERAL RURAL DO RIO DE JANEIRO
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

ATA Nº 217/2023 - PPGCV (12.28.01.00.00.00.00.50)

Nº do Protocolo: 23083.020836/2023-58

Seropédica-RJ, 05 de abril de 2023.

UNIVERSIDADE FEDERAL RURAL DO RIO DE JANEIRO
INSTITUTO DE VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

PRISCILLA NUNES DOS SANTOS

Tese submetida como requisito parcial para a obtenção do grau de Doutor(a) em Ciências, no Programa de Pós-Graduação em Ciências Veterinárias.

TESE APROVADA EM 23/06/2020

Conforme deliberação número 001/2020 da PROPPG, de 30/06/2020, tendo em vista a implementação de trabalho remoto e durante a vigência do período de suspensão das atividades acadêmicas presenciais, em virtude das medidas adotadas para reduzir a propagação da pandemia de Covid-19, nas versões finais das teses e dissertações as assinaturas originais dos membros da banca examinadora poderão ser substituídas por documento(s) com assinaturas eletrônicas. Estas devem ser feitas na própria folha de assinaturas, através do SIPAC, ou do Sistema Eletrônico de Informações (SEI) e neste caso a folha com a assinatura deve constar como anexo ao final da tese.

(Assinado digitalmente em 04/05/2023 15:01)
ADIVALDO HENRIQUE DA FONSECA
PROFESSOR DO MAGISTERIO SUPERIOR
DESP (12.28.01.00.00.00.00.52)
Matrícula: ###58#7

(Assinado digitalmente em 03/05/2023 10:43)
HUARRISSON AZEVEDO SANTOS
PROFESSOR DO MAGISTERIO SUPERIOR
DESP (12.28.01.00.00.00.00.52)
Matrícula: ###014#2

(Assinado digitalmente em 03/05/2023 21:08)
MARISTELA PECKLE PEIXOTO
PROFESSOR DO MAGISTERIO SUPERIOR
DeptPA (12.28.01.00.00.00.00.55)
Matrícula: ###034#8

(Assinado digitalmente em 13/06/2023 09:11)
BRUNA DE AZEVEDO BAÊTA
ASSINANTE EXTERNO
CPF: ###.###.697-##

(Assinado digitalmente em 03/05/2023 10:40)
ERICH PETER ZWEYGARTH
ASSINANTE EXTERNO
Passaporte: ###9PGT#T

DEDICATION

I dedicate this thesis to my beloved grandmother Noêmia Maria dos Santos (*in memoriam*) who was a great supporter of my dreams.

ACKNOWLEDGEMENTS

To the superior force that, for those who believe, brings motivation and is emanated by each one of us in some way.

To my parents Adriana Nunes dos Santos and Marcos Cesar dos Santos, and also to my brother Marcos Cesar Nunes dos Santos for all love, words of motivation, and prayers.

To my fiancé Luís Carlos Leal for understanding, love, and for always supporting my growth.

To my longtime friends Queila Maria de Souza, Arielle Jayme, and Camila Igne Rocha for the good and light moments together (even if rare).

To my supervisor Adivaldo Henrique da Fonseca, for being a model of diplomacy and resilience, for his supervision and for always encouraging me to face adversity. Also, to Professor Marília Massard da Fonseca for always being so kind and receiving us so well in her home.

To Dr. Erich Peter Zweygarth, for all the shared experience and for his precious friendship. For being an example of modesty and competence among experienced professionals in the academic environment.

To Michele Bahia, for always being available to help, for all her help collecting blood and parameters from the cattle, in addition to being a friend and supporter.

To Juliana Ferreira, for always being available for sample collection, support in cell maintenance, and for being a reliable and friendly person.

To Matheus Cordeiro, for having done many sample collections, in addition to supporting in the molecular biology part and always being willing to answer questions.

To Professor Maristela Peckle Peixoto, who dedicated herself to building the phylogenetic tree.

To my laboratory colleagues José Luis Bautista, Adlilton Pacheco, Marcio Barizon, Carolina Marotta, Jaqueline Valim, Neide Gomes, Thays Figueiroa, Priscila Vieira, Letícia Leitão, and Yasmin Kaulich for sharing good moments, and for the help and friendship.

To my co-supervisor Claudia Bezerra da Silva, who always offers solutions, answers to questions, and participates in field activities, and for being an example of a professional and a person.

To my co-supervisor Jenevaldo Barbosa da Silva, who has supported, and taught topics related to hemoparasites to me since I was an undergraduate, and for assuaging my doubts and for being the epitome of dedication and professionalism.

To Professor Itabajara Vaz, for offering the Porto Alegre strain ticks used in this study.

To Adenir Alves, for always being committed to the care of animals, ensuring their well-being.

To the experimental animals Gordo and Natal, that were always nice to deal and provided the results of this thesis.

To my cat Shakiro, for the company during the writing in Seropédica.

To INIAV, for receiving me during the sandwich PhD and to make it possible to carry out the second chapter of this thesis.

To Dr. Jacinto Gomes, my supervisor during the sandwich doctorate (PDSE), for his patience, sharing his professional experience, and for encouraging me during the attempts to isolate *Anaplasma* sp.

To Professor Ana Maria Munhoz, for being so attentive, dear, and friendly after my arrival in Lisbon, and for cooperating in the supply of samples for the isolation attempts.

To Dr. Helga Waap, the technicians Mrs. Maria and Lucinda, and the master's degree students Lara Augusto and Pedro Subtil for the companionship, encouragement, and sharing of experiences.

To the virology department colleagues Dr. Miguel Fevereiro, Dra. Margarida and Dra. Silvia for offering their space to work on cell maintenance, for sharing experiences, and for providing material for the cultivation attempts.

To veterinarian Miguel Minas, who made possible the last collection of samples in Alentejo.

To the technicians Ms. Conceição, Filomena, and Helena for their patience and for always helping me in the culture medium preparation.

To Mrs. Umbelina and Alexandrina for the care and affection during my stay at INIAV Residence.

To my friend Abdul Muktadir Shafi for his support, for being a good friend, and for always bringing words of comfort and good times.

To my friend Lee for sharing such happy moments.

To my colleagues at the Residence, Abdulbasit Amin, Mehran Piran, Helena Almeida, Joaquina, Inácio Filipe, Nzengele Nzambi and Adão Gonçalves, for the good times.

To my Brazilian colleagues Luciano Pereira, Talítha Gorgulho, Catiele, Priscila Verona, Carolina, Lívia Maria, and Júnior Abalos, who were present at such happy moments in Lisbon.

To Izabela Mesquita Araújo and her mother Maria da Cruz Mesquita, for their help and altruistic participation in the execution of my work. Also, to Letícia Vicente for her absolutely free-of-charge helpfulness, and to João Antônio Lima and Juliana Hamoy for offering the possibility of fundamental help in a mishap-ridden time in my research. Your help was ESSENTIAL and I will never forget your contributions.

To the Graduate Program of Veterinary Sciences, for the support provided to carry out the project.

To UFRRJ, for being a place where I shared experiences, lived and learned things that went beyond technical knowledge.

To the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the scholarship granting for the Sandwich PhD Abroad scholarship (PDSE).

O presente trabalho foi realizado com o apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brasil (CAPES) - Código de Financiamento 001, agradeço o financiamento desta pesquisa.

GENERAL ABSTRACT

SANTOS, Priscilla Nunes dos. Diversity of the *Anaplasma marginale msp*1α gene and isolation and propagation of a novel genotype of *Anaplasma* sp. in IDE8 cells. 2020.106p. Thesis (PhD in Veterinary Sciences). Veterinary Institute, Department of Epidemiology and Public Health, Federal Rural University of Rio de Janeiro, Seropédica, RJ, 2020.

This thesis consists of two chapters: The purpose of the first chapter was to evaluate the tandem repeat diversity in the major surface protein MSP1a of *Anaplasma marginale* in experimentally infected cattle and in *Rhipicephalus microplus* over the span of 1 year, and the re-isolation of AmRio1 strain of *A. marginale*, using the buffy coat separation protocol, was performed. The second chapter was aimed at isolating, propagating, and characterizing a novel *Anaplasma* sp. genotype from Portugal in IDE8 cells. To evaluate *A. marginale* genetic diversity, cattle were infested with *R. microplus* larvae and subsequently infected by *A. marginale* from tick cell cultures. The strains AmRio1 and AmRio2 were inoculated in bovine 1 and bovine 2, respectively. Subsequently, blood samples were collected over 1 year for blood smears, hematocrit, plasma proteins, and semi-nested polymerase chain reaction (PCR) for the *msp*5 and *msp*1α genes. The buffy coat separation protocol was used to isolate *Anaplasma* sp. Ticks were collected and their salivary glands and guts DNA were tested using semi-nested PCR for the *msp*5 and *msp*1α genes. Both animals showed stability in the evaluated parameters and absence of clinical signs, showing that they had persistent infection. A blood smear from Animal 1 showed inclusions inside the leukocytes, and four attempts were made to isolate *Anaplasma* sp. using the buffy-coat as inoculum. Animal 1 became positive for the *msp*5 gene on day 12 after infection, while positivity was detected in Animal 2 only 105 days after first exposure to the agent. The tandem repeats were stable when the blood and tick samples of both animals were evaluated, demonstrating the presence of the strains AmRio1 in Animal 1 and AmRio2 in Animal 2. In the second chapter, erythrocytes from a naturally infected bovine from Portugal were used for isolating *Anaplasma* sp. in IDE8 cells. Suggestive inclusions of infection were observed in the IDE8 cells 35 days after inoculation. The Anaplasmataceae family 16S rRNA gene

from the cultures was sequenced and showed that the isolated rickettsia was close to the *A. platys* species in the phylogenetic tree.

Keywords: Diversity, *Rhipicephalus microplus*, IDE8, *msp*1α.

RESUMO GERAL

SANTOS, Priscilla Nunes dos. Diversidade do gene *msp*1α de *Anaplasma marginale* e isolamento e propagação de um novo genótipo de *Anaplasma* sp. em células IDE8. 2020. 106p. Tese (Doutorado em Ciências Veterinárias). Instituto de Veterinária, Departamento de Epidemiologia e Saúde Pública, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2020.

Esta tese consiste em dois capítulos: O primeiro capítulo teve como objetivo avaliar a diversidade das repetições em tandem da proteína principal de superfície MSP1a de *Anaplasma marginale* em bovinos infectados experimentalmente e em *Rhipicephalus microplus* durante o período de 1 ano e foi realizado o re-isolamento da estirpe AmRio1 de *A. marginale* a partir de leucócitos bovinos. O segundo capítulo teve como objetivo isolar, propagar e caracterizar um novo genótipo de *Anaplasma* sp. oriundo de Portugal em células IDE8. Para avaliar a diversidade genética de *A. marginale*, dois bovinos foram infestados com larvas de *R. microplus* e posteriormente infectados por *A. marginale* de culturas de células de carrapatos. As cepas AmRio1 e AmRio2 foram inoculadas nos bovinos 1 e 2, respectivamente. Posteriormente, amostras de sangue foram coletadas por um ano para esfregaços de sangue, hematócrito, proteínas plasmáticas e Reação em Cadeia da Polimerase semi-nested (semi-nested PCR) para os genes *msp*5 e *msp*1α. *Anaplasma marginale* também foi isolado dos leucócitos bovinos. Os carrapatos foram coletados e DNA de suas glândulas salivares e intestinos foram testados usando semi-nested PCR para os genes *msp*5 e *msp*1α. Ambos os animais apresentaram estabilidade nos parâmetros avaliados e ausência de sinais clínicos, demonstrando que apresentaram infecção persistente. Um esfregaço de sangue do Animal 1 mostrou inclusões em leucócitos e foram feitas quatro tentativas para isolar *Anaplasma* sp. a partir de leucócitos. O animal 1 tornou-se positivo para o gene *msp*5 no dia 12 após a infecção, enquanto a positividade foi detectada no animal 2 apenas 105 dias após a primeira exposição ao agente. As repetições em tandem permaneceram estáveis quando avaliadas as amostras de sangue e carrapato de ambos os animais, demonstrando a presença das estirpes AmRio1 no Animal 1 e AmRio2 no Animal 2. No segundo capítulo, foram utilizados eritrócitos de um bovino naturalmente infectado em Portugal para isolar *Anaplasma* sp. em células IDE8. Inclusões sugestivas de infecção foram observadas nas células IDE8 35

dias após a inoculação. O gene 16S rRNA da família Anaplasmataceae das culturas foi sequenciado e mostrou que a rickettsia isolada estava próxima da espécie *A. platys* na árvore filogenética.

Palavras-chave: Diversidade, *Rhipicephalus microplus*, IDE8, *msp*1α.

LIST OF TABLES

CHAPTER I

LIST OF FIGURES

LITERATURE REVIEW

LIST OF FIGURES

CHAPTER I

CHAPTER II

SUMMARY

1 GENERAL INTRODUCTION

Bovine anaplasmosis is a disease caused by the agent *Anaplasma marginale*. It has economic importance because it decreases cattle productivity, particularly in dairy farms (KOCAN et al., 2010; MACHADO et al., 2015). The disease is endemic in the tropical and subtropical regions of the world and is transmitted mechanically by fomites, the mouth parts of hematophagous diptera and ticks contaminated with blood infected by the bacterium (KOCAN et al., 2004b), biologically by ticks, and vertically by the transplacental route (ABBA et al., 2016; ZABEL et al., 2018).

The only known site of *A. marginale* replication in cattle is the erythrocytes (ALLEMAN et al., 1997). Once infected, the number of affected cells grows logarithmically and may lead to clinical signs (KOCAN et al., 2010). Cattle that survive the acute infection develop persistent infection, and become reservoirs with low cyclic rickettsemia, serving as a source of infection to susceptible animals (KOCAN et al., 2010).

The *A. marginale* major surface proteins (MSPs) have sequence variations and have an evolutionary history probably influenced by the pathogen–vector interaction (ESTRADA-PENA et al., 2009). One of these proteins, MSP1a, is considered a stable genetic marker for the identification of geographical strains, presenting variation among them (ALLRED et al., 1990; DE LA FUENTE et al., 2007b; ESTRADA-PENA et al., 2009); the same has been observed in the microsatellite located in the 5′ untranslated region (5′UTR) in this protein (ESTRADA-PENA et al., 2009). MSP1a is encoded by the *msp1α* gene, and is involved in the bacterium's adhesion to bovine erythrocytes and tick cells (MCGAREY et al., 1994a; DE LA FUENTE et al., 2001c; SANTOS et al., 2019). The genetic characteristics of this protein include the encoding of tandem repeats for amino acids in the N-terminal region domain, which shows high mutability compared to the rest of the protein (BOWIE et al., 2002). It is believed that this protein has evolved due to pressures from the host immune system and that it co-evolved with the tick vector, undergoing selection due to characteristics related to the pathogen–vector interaction (DE LA FUENTE et al., 2007b). It is also considered a substrate for vaccine production because it is a B cell epitope.

The first tick cell lines were established 50 years ago. These cell lines have been applied extensively to isolate, propagate, and study tick-borne viruses and bacteria and also for conducting tick–pathogen interaction studies (BELL-SAKYI et al., 2007; BELL-SAKYI et al., 2018). The ability of tick cells to survive for many months without subculturing facilitates the isolation of bacterial species that are fastidious or very slow-growing for isolation, which can occur in cases where the inoculum has an extremely low amount of bacteria (BELL-SAKYI et al., 2018). Since 1995, many members of the *Anaplasma* and *Ehrlichia* genera have been established in tick cell lines. These studies have contributed to knowledge of the species that make up these genera and their evolutionary relationships, as new species have been isolated, propagated, and described (BELL-SAKYI et al., 2018).

The results of this thesis are presented in two chapters: The first refers to the original project that aimed to characterize *A. marginale* strains by analyzing the MSP1a tandem repeats in persistently infected cattle and in *Rhipicephalus microplus* ticks from the infestation of these animals. In the same chapter, *A. marginale* was isolated from the leukocyte layer from the blood of Animal 1. The second chapter is the result of a project carried out during a sandwich PhD at the National Institute of Agricultural and Veterinary Research (INIAV) in Oeiras, Portugal. In that chapter, an organism belonging to the family Anaplasmataceae, provisionally termed *Anaplasma* sp., was isolated, propagated, and characterized.

2 LITERATURE REVIEW

2.1 *Anaplasma marginale*

2.1.1 History

On their exceptional work, Smith and Killborne (1893) discovered that ticks were vectors of Texas cattle fever. However, they mistakenly concluded that the "marginal points" they observed were part of the biological cycle of *Babesia bigemina* (SMITH; KILLBORNE., 1893; KOCAN et al., 2003). Another relevant historical record was published by Salmon and Smith (1896). They described a dot-type pathogen in bovine blood smears as "tiny blue-colored corpuscles, always located at the edge of the cell" (KOCAN et al., 2003).

Arnold Theiler also described the genus *Anaplasma* in South African cattle erythrocytes as "marginal points" (THEILER, 1910). He determined that babesiosis and bovine anaplasmosis were different diseases and that they often coexisted in the same animal. Subsequent publications proved that the animals studied by Smith and Kilborne were simultaneously infected with *Anaplasma* and *Babesia* (KOCAN et al., 2003). Theiler also described two subspecies: *A. marginale*, observed on the margins of the cells, and *A. centrale*, which apparently was less pathogenic and observed in the center of erythrocytes (THEILER, 1911).

2.1.2 Taxonomy

A reorganization of the Rickettsiales order was proposed by Dumler et al. (2001) based on a comparison of the 16S rRNA gene (coding for the smallest subunit of the ribosome), the groESL gene (encoding the heat shock protein), and surface proteins. According to Dumler et al. (2001), *A. marginale* belongs to the order Rickettsiales, family Anaplasmataceae, and genus *Anaplasma*, together with the species *A. platys*, *A. bovis*, *A. ovis*, *A. centrale*, and *A. phagocytophilum*.

2.1.3 Morphology

The genera *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia*, all belonging to the Anaplasmataceae family, include intracellular bacteria, which are found in eukaryotic cell vacuoles (RYMASZEWSKA; GRENDA, 2008). *Anaplasma* spp. are small, gram-negative bacteria, often pleomorphic, coccoid to ellipsoidal, and always found in cytoplasmic or parasitophorous vacuoles. They form compact inclusions (morulae) in the mature or immature hematopoietic cells of vertebrate hosts (DUMLER et al., 2001).

Inclusion corpuscles are found on the periphery of erythrocytes and have 4–8 rickettsiae (Figure 1), while in the infected ticks cells, there are colonies containing hundreds of organisms (Figure 2) (KOCAN et al., 2003). Ultrastructural analysis has revealed larger cross-linked cells and smaller forms with condensed protoplasm, termed dense forms (POPOV et al., 1998). The cross-linked forms divide by binary fission and subsequently become dense forms, which are infectious and can survive outside the cell for a limited time (Figure 3) (KOCAN et al., 2010).

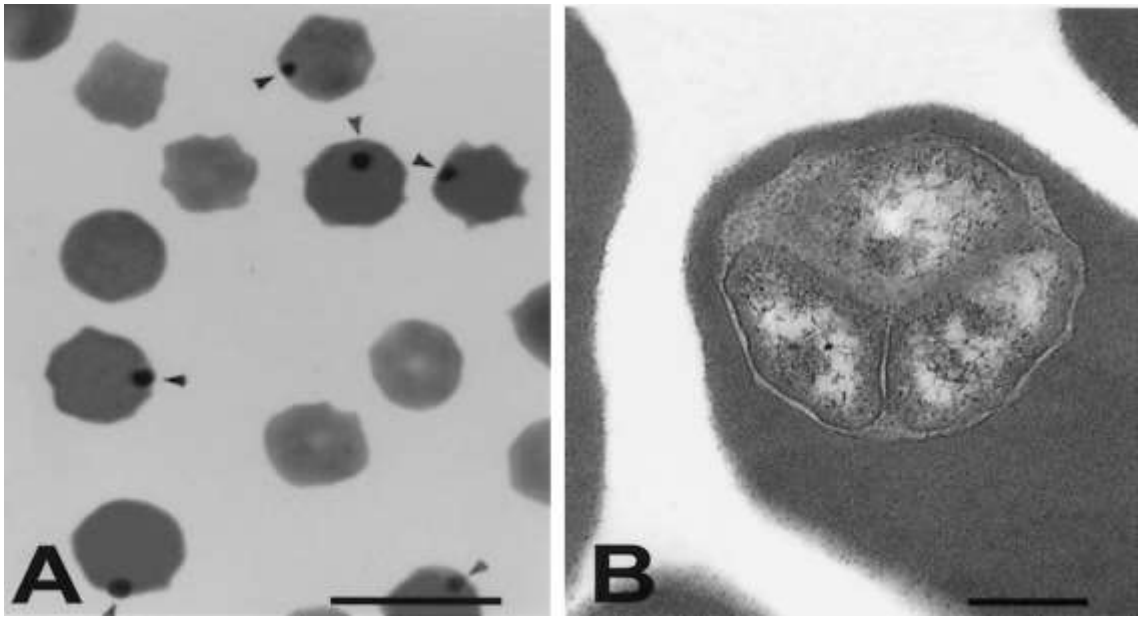

Figura 1: Bovine erythrocytes infected by *A. marginale*. (A) Inclusion corpuscles (arrowheads) on the periphery of an erythrocyte in a blood smear. (B) Electron microscopy of an *A. marginale* inclusion with three organisms. Bar = 10 µm (A) and 0.5 µm (B). Source: KOCAN et al. (2003).

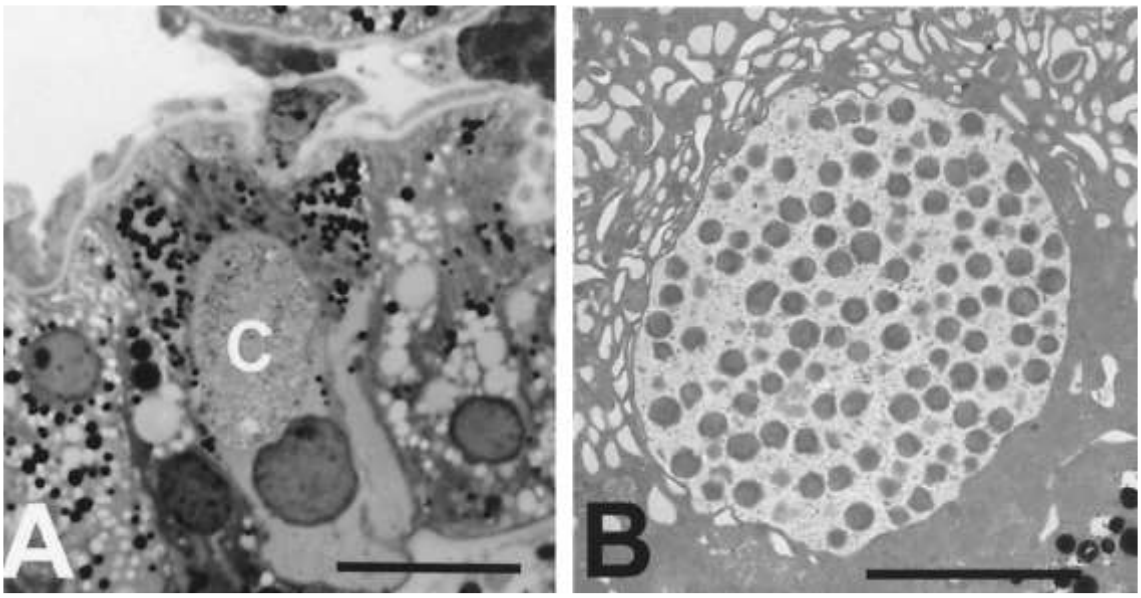

Figure 2: *Anaplasma marginale* colonies in tick gut cells. (A) Light microscopy of a large colony (C) in a tick gut cell. (B) Electron microscopy of a colony in a tick gut cell. Bar = 10 µm (A) and 5 µm (B). Source: KOCAN et al. (2003).

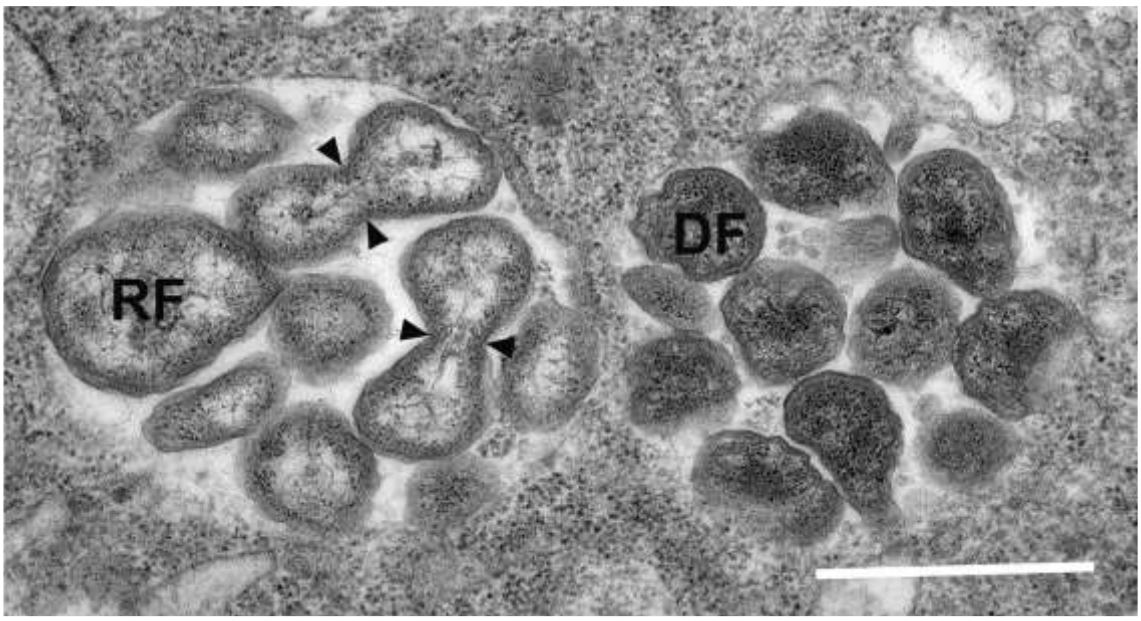

Figure 3: Electron microscopy of the two developmental stages of *A. marginale* within colonies in tick cells. Reticulated forms (RF) inside a colony dividing by binary fission (arrows) and transforming into dense forms (DF). Scale = 1 µm Source: BLOUIN et al. (2002).

2.1.4 Developmental cycle of *A. marginale* in cattle and in tick cells

Erythrocytes are the only known infection site of *A. marginale* in cattle (KOCAN et al., 2010). Munderloh et al. (2004) reported the development of *A. marginale* in an endothelial cell line, but no endothelial cell infections were observed *in vivo*.

The biological cycle of *A. marginale* is complex and coordinates with the tick's feeding cycle (KOCAN, 1986; KOCAN et al., 1992a, b) . Infected erythrocytes are ingested and work as a source of infection for tick gut cells (KOCAN et al., 2003). After *A. marginale* growth in the tick gut cells, many other tissues become infected, including the salivary glands, from where rickettsiae are transmitted to vertebrate hosts during the blood meal (KOCAN, 1986; KOCAN et al., 1986, 1992a, b; GE et al., 1996). By infecting tick tissues, *A. marginale* develops inside vacuoles or membranes inside cells (KOCAN et al., 2003).

The first form of *A. marginale* observed inside vacuoles is reticulate (vegetative), which divides by binary fission, forming large colonies containing hundreds of organisms (KOCAN et al., 2004). The reticulated form suffers a change, giving rise to the dense form, which is infectious, and can survive outside the tick host cells. Cattle become infected when the dense form is transmitted via saliva during tick feeding (KOCAN et al., 2004).

After erythrocyte infection, initial corpuscles are observed inside the cells, reaching up to 70% of infected erythrocytes during acute infection (RISTIC et al., 1977; RICHEY, 1981). Erythrocytes infection increases in geometric progression (KOCAN et al., 2010). The pre-patent period varies according to the infecting dose and can last between 7 and 60 days, with an average of 28 days. The infected erythrocytes are subsequently phagocytosed by the animal's reticuloendothelial cells, resulting in the development of mild to severe anemia and jaundice without hemoglobinemia and hemoglobinuria (KOCAN et al., 2010).

The clinical signs include fever, weight loss, miscarriage, lethargy, jaundice, and frequently, death, in animals over 2 years old (RISTIC et al., 1977). Cattle that survive an acute infection develop persistent infections characterized by low levels of rickettsemia (FRENCH et al., 1998, 1999; KIESER et al., 1990). "Reservoirs hosts" or persistently infected animals, have long-lasting immunity, and under the challenge of exposure, do not develop the clinical disease (KOCAN et al., 2010).

2.1.5 Transmission

There are three forms of transmission of *A. marginale*: biologically by ticks; mechanically by hematophagous flies or by fomites contaminated with infected blood; and by the transplacental route (THEILER, 1910; DICKMANS, 1950; ZAUGG et al., 1985; KOCAN et al., 2003; ESTRADA-PENA et al., 2009).

2.1.5.1 Biological transmission

Biological transmission occurs when vector ticks feed on erythrocytes infected with *A. marginale*. After the infected blood is ingested, the agent replicates in the tick gut cells and salivary glands and is transmitted via saliva to susceptible animals (AUBRY et al., 2011).

Approximately 20 species of ticks are considered vectors worldwide (EWING, 1981). Ticks capable of transmitting *A. marginale* include *Dermacentor* spp., *Ixodes ricinus*, and *Rhipicephalus* spp., while *Amblyomma* spp. has not been shown to be a vector of *A. marginale* (KOCAN et al., 2004a).

Vector species are capable transstadial transmission (from stage to stage) and intrastadial transmission (within a given stage), while transovarial transmission has not been observed (STICH et al., 1989; KOCAN et al., 2010). *Anaplasma marginale* is transmitted intrastadially by male ticks (KOCAN et al., 2010). These ticks serve as reservoirs for *A. marginale*, as they become persistently infected by the pathogen; the same occurs with persistently infected cattle (KOCAN et al., 1992; GE et al., 1996; PALMER et al., 2001). Male ticks can move from one animal to another in their search for females (ZAUGG et al., 1986). Infected males can be an important mechanism of transmission of *A. marginale* in host vectors, such as species of the subgenus *Rhipicephalus* and *D. albipictus* (KOCAN et al., 2010).

Information on the prevalence of *A. marginale* in ticks in Europe is still scarce (DE LA FUENTE et al., 2004c). *Hyalomma marginatum*, *R. bursa*, and *D. marginatus* are the most abundant species of ticks collected, being the species most commonly found in ungulates in Mediterranean countries (GILOT et al., 1985). A

recent study revealed the participation of *R. bursa* in the transmission of *A. marginale* in Portugal (FERROLHO et al., 2016). DE LA FUENTE et al. (2004a) suggested in a study in central Spain that other ticks such as *D. marginatus* and *H. marginatum*, not yet recognized as vectors, could play a role in *A. marginale* transmission, even between wild and native species.

In tropical and subtropical areas, the ticks mainly involved in transmitting *A. marginale* are *Dermacentor* spp. (*D. andersoni*, *D. variabilis*, *D. albipictus*) and *Rhipicephalus* spp. (*R. microplus* and *R. annulatus*) (KOCAN et al., 2015).

Some species of ticks are capable of becoming infected even when they feed on persistently infected cattle (SCOLES et al., 2005). Infection levels can reach 10^4–10^5 organisms per salivary gland, regardless of the level of rickettsemia in the blood during the blood meal period (ERIKS et al., 1993).

2.1.5.2 Mechanical transmission

Mechanical transmission can occur via fomites contaminated with infected blood and by blood-sucking diptera (DIKMANS, 1950; KOCAN, 1986; KOCAN et al., 2003; KOCAN et al., 2004b). Fomites contaminated with infected blood include needles, dehorning instruments, nose rings, tattoo instruments, earrings, and castration instruments (KOCAN et al., 2003). The arthropods involved in mechanical transmission are hematophagous diptera of the genera *Tabanus* and *Stomoxys*, and mosquitoes (EWING, 1981; POTGIETER et al., 1982; FOIL, 1989).

Although it does not have the same transmission efficiency, as noted by SCOLES et al. (2005) when comparing transmission efficiency from both animals with acute infection and animals with chronic infection, mechanical transmission plays an important role in the spread of *A. marginale*, especially in areas where vector ticks do not seem to exist, as observed in some areas of South and Central America (EWING, 1981; FOIL, 1989; BASTOS et al., 2009; YANG et al., 2016) and in areas where *R. microplus* does not seem to act as a biological vector for *A. marginale* (FIGUEROA et al., 1998; FUTSE et al., 2003).

In addition to the locations where vector ticks are absent, mechanical transmission acts as the only means of transmitting *A. marginale* in regions where

ticks are unable to transmit local strains, as in the case of the Florida isolate of *A. marginale* that has been proven non-infective for ticks (KOCAN et al., 2015).

2.1.5.3 Transplacental transmission

Transplacental transmission occurs during pregnancy from cow to calf (AUBRY et al., 2011). This form of transmission results in healthy, but persistently infected calves (KOCAN et al., 2015). Experimentally, vertical transmission was observed only in the second and third months of pregnancy (ZAUGG, 1985). Transplacental transmission was also observed in calves born from animals that contracted clinical anaplasmosis in the last 2 months of pregnancy (SALABARRIA et al., 1988). A study in South Africa reported a transplacental transmission of 15.6% (POTGIETER et al., 1987). Therefore, transplacental transmission plays an important epidemiological role in some regions (KOCAN et al., 2003).

2.1.6 Distribution

Bovine anaplasmosis occurs in tropical and subtropical countries worldwide, and is the main disease affecting cattle production in many countries (KOCAN et al., 2010). The disease is enzootic in the USA and in all Latin American countries, with the exception of mountainous and desert areas, such as the Andes (GUGLIELMONE, 1995; KOCAN et al., 2010). In Europe, *A. marginale* is found mainly in Mediterranean countries in cattle and wild species (DE LA FUENTE et al., 2005). The disease is also endemic in Asia and Africa (KOCAN et al., 2010). The distribution of anaplasmosis will likely continue to change as a result of global warming, which is expected to influence the movement of vector ticks (JONSSON et al., 2000).

2.1.7 Vertebrate hosts

Anaplasma marginale is an agent of great importance in cattle breeding, but it can also infect other ruminants (AUBRY et al., 2011). Young animals appear to be less susceptible to infection, and when infected, they are less susceptible to clinical disease (KOCAN et al., 2010). After acute infection has occurred, they become persistently infected, and have a low degree of parasitemia (FRENCH et al., 1998).

Carrier animals are a source of infection for mechanical and/or biological transmission. Cattle breeds of the subspecies *Bos taurus taurus* (e.g. Dutch, Brown Swiss, or Hereford) are more susceptible to developing acute anaplasmosis than crossbred Zebu animals (AGUIRRE et al., 1994; GUGLIELMONE, 1995; ARAUJO et al., 2003). Clinical anaplasmosis occurs most often in cattle, but other ruminants, including buffalo (*Bubalus bubalis*), American bison (*Bison bison*), white-tailed deer (*Odocoileus hemionus hemionus*), black-tailed deer (*O. hemionus columbianus*) and rocky mountain deer can become infected with *A. marginale* (KUTTLER, 1984; ZAUGG et al., 1996) .

Concerns about the transmission of infectious agents between wildlife and cattle herds are growing, especially where they share the same pasture areas (CHOMEL et al., 1994). With the exception of two reports of acute anaplasmosis in giraffes, naturally infected animals showing an apparent clinical picture have not been reported (DAVIDSON et al., 2001; SCOLES et al., 2008). DE LA FUENTE et al. (2004a) observed 10% seroprevalence in Iberian red deer in southeastern Spain, in the Castilla–La Mancha region. As observed in black-tailed deer, red Iberian deer had subclinical infections, being considered reservoirs for *A. marginale* infection in some regions (BOYNTON et al., 1933; CHRISTENSEN et al., 1960; KUTTLER, 1984; DE LA FUENTE et al., 2004a).

In Europe, the infection of wild ungulates by *A. marginale* has also recently been observed. (PEREIRA et al., 2016). The ticks *H. marginatum* and *R. bursa* feed on deer and cattle, thus enabling *A. marginale* transmission by these species among these animals (KOCAN et al., 2004b). The species *H. marginatum* and *R. bursa*, found in red Iberian deer in southeastern Spain, had a prevalence of *A. marginale* infection of 39% and 20%, respectively (DE LA FUENTE et al., 2004a). Thus, wild ruminants could play a role in the epidemiology of *A. marginale* in some regions.

2.1.8 Diagnosis of anaplasmosis

When observing the clinical signs, the first sign of anaplasmosis is commonly pyrexia, and it can occur before the infection reaches 1% of the erythrocytes. Fever above 40°C usually persists during the period of rising parasitemia, with abnormally high body temperatures until death, in terminal cases (KOCAN et al., 2010). A specific feature of anaplasmosis is anemia associated with phagocytosis of erythrocytes. The severity of clinical signs is associated with the degree of anemia and includes pallor of the skin and mucous membranes, and increased heart and respiratory rates. The corpuscular volume decreases, coinciding with the increase in parasitemia. Animals become weak, anorexic, and lethargic. Cows can abort, and have decreased milk production. In buffaloes, temporary infertility has been observed (JONES et al., 1966; KOCAN et al., 2010).

In severe cases of the disease, cattle develop gastrointestinal atony, ruminal stasis, and constipation that is associated with dehydration and weight loss. Some animals have neurological deficits due to episodes of cerebral anoxia. Jaundice usually occurs at the end of the disease and is most commonly observed during early convalescence. Recovery is more common in young animals. Mortality rates of 50–60% are described in adult animals, especially when older animals are stressed. The occurrence of death in cattle while the animals are being prepared for treatment is not uncommon. The expected necropsy findings from clinical signs include severe anemia, jaundice, splenomegaly, and hepatomegaly. Petechial hemorrhages are often seen on serous surfaces, especially in the heart and pericardium, showing a pale and flabby heart (COETZEE et al., 2005).

Blood smears stained using the Giemsa or Diff-Quick method allow the observation of *A. marginale* inside the erythrocytes (KOCAN et al., 2010). However, optical microscopy is ineffective when parasitemia is very low and before clinical signs become apparent, or in persistently infected cattle (KOCAN et al., 2015). During these periods, the inclusions can be easily confused with Howell-Jolly bodies, basophilic stippling of immature erythrocytes, and artifacts from staining (KOCAN et al., 2015).

One form of laboratory diagnosis is competitive enzyme-linked immunosorbent assay (cELISA) based on the *A. marginale* surface protein MSP5. This protein in principle is considered to have high sensitivity and specificity.

However, cross-reactions with antibodies from other organisms of the genus *Anaplasma* have been detected due to the conservation of the *msp5* gene (KOCAN et al., 2012); (KOCAN et al., 2015). Although DNA-based diagnoses are species-specific, they are not practical for assessing prevalence on a large scale (KOCAN et al., 2010): (KOCAN et al., 2015).

The polymerase chain reaction (PCR) was also developed to detect the presence of a low level of infection in persistently infected cattle (AUBRY et al., 2011). A more sensitive nested PCR (nPCR) could detect less than 30 infected red blood cells per milliliter of blood (the equivalent of 0.0000001% parasitemia) under experimental conditions, which could be below the reduced levels found in persistently infected cattle (TORIONI DE ECHAIDE et al., 1998). PCR targeting the *msp*4 and/or *msp*1α genes have been used to differentiate isolates from *A. marginale*, which is useful for tracing the origin of an outbreak and for differentiating species of *Anaplasma* such as *A. marginale* and *A. centrale* (DE LA FUENTE et al., 2001c); (LEW et al., 2002).

2.2 Major surface proteins

The *A. marginale* genome is circular and is approximately 1.2–1.6 Mb in size (ALLEMAN et al., 1993; BARBET, 1995; SATO et al., 2009). Of 949 *A. marginale* coding sequences, 62 are from external membrane proteins; of these, 49 belong to the MSPs of the *msp*1 and *msp*2 superfamilies (KOCAN et al., 2010). Six MSPs derived from bovine erythrocytes have been identified, and information on gene sequence, recombinant proteins, monospecific and monoclonal antibodies, isolate variability, potential value in diagnostic assays, and vaccine availability has been obtained (KOCAN et al., 2003). Three of these MSPs, named MSP1a, MSP4, and MSP5, are derived from unique genes and do not vary antigenically in the same strain, while the other three, i.e., MSP1b, MSP2, and MSP3, are from multigene families and can vary antigenically, most notably in persistently infected cattle (KOCAN et al., 2003).

MSP1a and MSP1b form the MSP1 complex. MSP1a is a functionally important protein with characteristics that contribute to its ability to act as a genetic marker for taxonomic studies of strain diversity (CABEZAS-CRUZ et al., 2015). MSP1b is encoded by at least two genes, *msp*1β1 and *msps*1β2; it is also polymorphic

among *A. marginale* geographical isolates (BARBET et al., 1987; CAMACHO-NUEZ et al., 2000; VISESHAKUL et al., 2000; BROWN et al., 2002). Although MSP1b is encoded by a multigenic family, only small variations in the MSP1b1 and MSP1b2 protein sequences have been observed during the lifecycle of the bacteria in cattle and ticks (UILENBERG, 1993; BOWIE et al., 2002; KOCAN et al., 2003). This protein is an adhesin for bovine erythrocytes (MCGAREY et al., 1994a); (MCGAREY et al., 1994a), but not for tick cells (DE LA FUENTE et al., 2001b).

MSP2 and MSP3 are encoded by large, polymorphic gene families (ALLEMAN et al., 1997); (PALMER et al., 1994). The sequence and antigenic composition of MSP2 varies during the rickettsemia cycle in cattle (BARBET, 1995; FRENCH et al., 1998; FRENCH et al., 1999; BASTOS et al., 2015) and in persistently infected ticks (DE LA FUENTE et al., 2010). The variation in the expression of these proteins allows the infection to persist in animals (PALMER et al., 2013).

MSP4 and MSP5 are highly conserved (DE LA FUENTE et al., 2002); (DE LA FUENTE et al., 2001a);(OBERLE et al., 1993); (DE LA FUENTE et al., 2003a); (TORIONI DE ECHAIDE et al., 1998). MSP4 provides phylogeographic information (DE LA FUENTE et al., 2010); (KOCAN et al., 2015); MSP5 has been used as an antigen in cELISA (TORIONI DE ECHAIDE et al., 1998).

2.2.1 MSP1a

MSP1a is used in many studies due to its role in the adhesion of *A. marginale* to bovine erythrocytes and tick cells, being determinant in bovine erythrocyte infection and bacterial transmission by ticks (CABEZAS-CRUZ et al., 2013a). In addition, it also plays a role in the development of immunity against *A. marginale* (AUBRY et al., 2011), and contributes to understanding of the genetic diversity of the agent within specific regions, heralding new concepts in relation to the evolution of pathogen–host interactions (DE LA FUENTE et al., 2007a); (DE LA FUENTE et al., 2004a); (DE LA FUENTE et al., 2005); (DE LA FUENTE et al., 2010); (ESTRADA-PENA et al., 2009).

The protein structure is preceded by a microsatellite in the 5′UTR region of the *msp1α* gene, identified as a regulator of the expression of the MSP1a protein

(ESTRADA-PENA et al., 2009). The microsatellite is located between the putative Shine-Dalgarno (SD) sequence (GTAGG) and the initiation codon (ATG). Its structure (bold text) is GTAGG **(G/A TTT)m (GT)n** T ATG (ESTRADA-PENA et al., 2009). The *A. marginale* genotype is determined by calculating the SD–ATG distance on the microsatellite (ESTRADA-PENA et al., 2009).

MSP1a is formed by a single polymorphic gene between strains, and is composed of a conserved region and a variable region, where the tandem repeats are found (ALLRED et al., 1990); (CABEZAS-CRUZ et al., 2015) (Figure 4). Its molecular weight varies between geographic isolates because of the variation in the number of repeated sequences or amino acids (23–31 amino acids) in the N-terminal portion of the protein (ALLRED et al., 1990); (DE LA FUENTE et al., 2005); (DE LA FUENTE et al., 2007a); (CABEZAS-CRUZ et al., 2013a); the C-terminal portion is highly conserved (ALLRED et al., 1990); (DE LA FUENTE et al., 2005); (DE LA FUENTE et al., 2007a). Three hundred and seventy-seven unique repeated sequences have been registered and deposited at GenBank (CABEZAS-CRUZ et al., 2015). With the advancement of studies on diversity, there is a tendency for this number to increase.

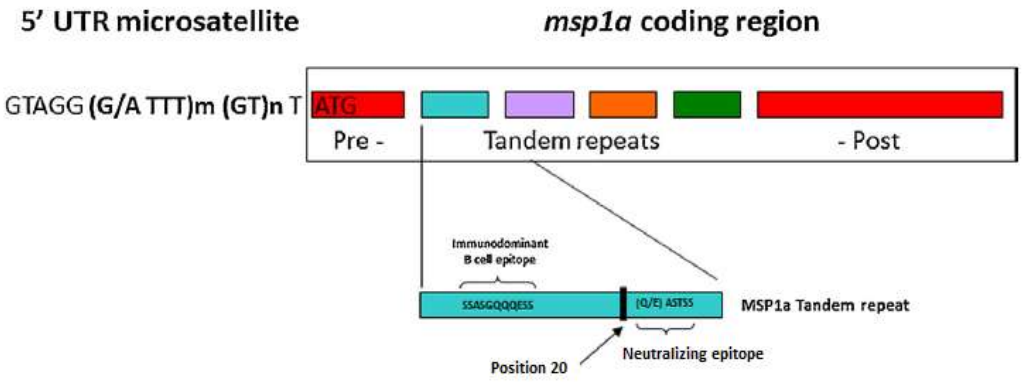

Figure 4: Schematic representation of MSP1a. The position and nucleotide sequence of the microsatellite in the 5′UTR is shown (capital letters), the number of "m" and "n" units varies between strains (ESTRADA-PENA et al., 2009). The pre-tandem (pre-), tandem (tandem repeats), and post-tandem (-post) regions are highlighted (colored boxes). One-letter codes are used to indicate the amino acid sequences of the immunodominant B cell epitope (GARCIA-GARCIA et al., 2004) and the neutralization epitope (ALLRED et al., 1990) as originally described. Position 20, related to the binding to the tick cell extract, is also demonstrated. Source: (CABEZAS-CRUZ et al., 2015).

Tandem repeats are peptides that differ in terms of amino acid amounts and composition (DE LA FUENTE et al., 2007a); (CABEZAS-CRUZ et al., 2013a). These structures are named using letters and numbers (DE LA FUENTE et al., 2007a) (Figure 5) and the combination of these tandem repeats constitute the variable region of the MSP1a protein of each strain (DE LA FUENTE et al., 2007a) (Figure 6). Most strains have more than one tandem repeat, and a maximum of 10 repeats. No strains have been observed with nine repeats (CABEZAS-CRUZ et al., 2013a).

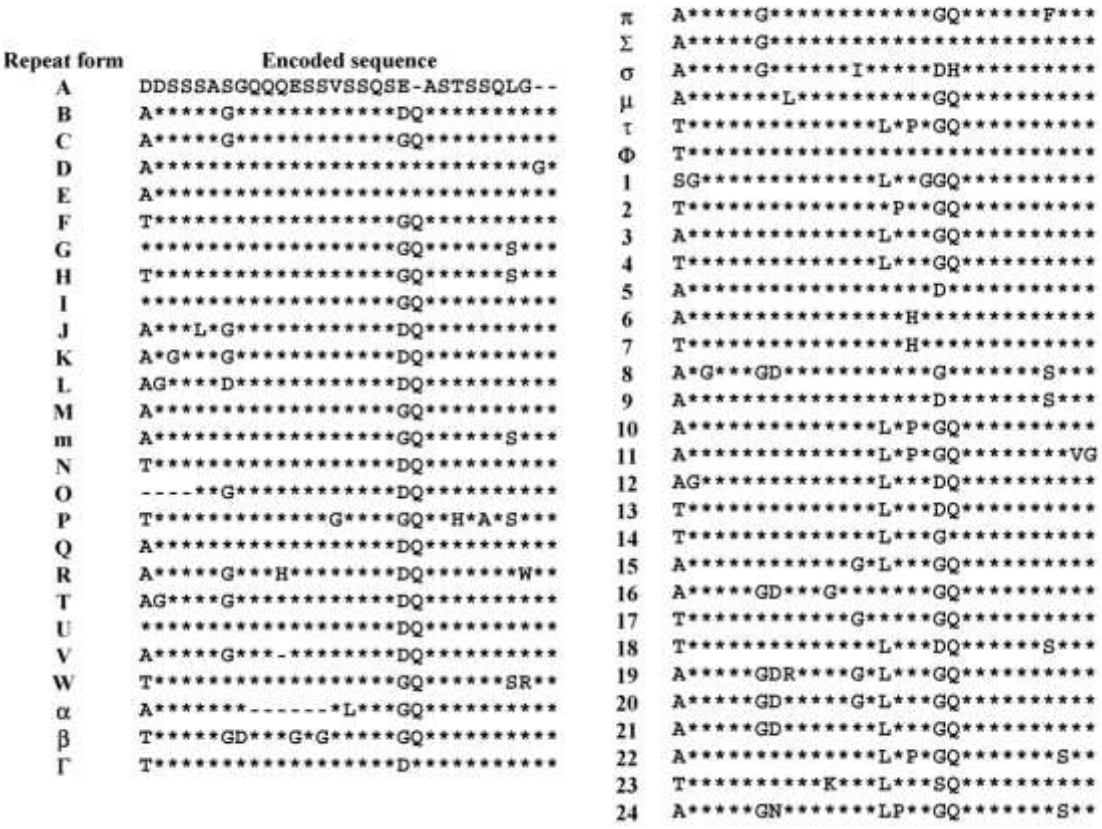

Figure 5: Sequences of tandem repeats in *A. marginale* strains of the sequence "A" to "24". The one-letter code represents the amino acids showing the different sequences found in the MSP1a tandem repeats. Asterisks indicate amino acids identical to those in the previous sequence; dashes indicate insertions/deletions. Source: DE LA FUENTE et al. (2007a) (adapted).

A. marginale strain	Country of origin	Genbank accession No.	Structure of MSP1a tandem repeats								No. of repeats
Florida	USA	M32871	A	B	B	B	B	B	B	B	8
Idaho	USA	M32868	D	D	D	D	D	E			6
Virginia	USA	AY010246	A	B							2
Washington	USA	M32869	B	B	B	C					4
Wetumka, OK	USA	AY010247	K	C	H						3
Cushing, OK	USA	AY127056	L	C	B	C					4
Cushing 2, OK	USA	AY127057	K	N	N	F	H				5
Glencoe 1, OK	USA	AY127053	K	F	N	F	H				5
Glencoe 2, OK	USA	AY127054	B	M	F	H					4
Glencoe 3, OK	USA	AY127055	T	B	C						3
Stillwater, OK	USA	AY127061	K	F	F	F	H				5
Stillwater 2, OK	USA	AY127062	L	B	C	C					4
Stillwater 68, OK	USA	DQ811776	K	B	M	F	H				5
Stillwater 483, OK	USA	DQ811777	K	B	M	H					4
Oklahoma City, OK	USA	AY127059	U								1
Okmulgee, OK	USA	AY127060	K	B	V	C					4
Stigler, OK	USA	AY127058	T	B	B	C					4
Pawhuska, OK	USA	AY127064	I	H							2
New Castle, OK	USA	AY127063	L	B	C	B					4
St. Maries, ID	USA	AY010245	J	B	B						3
California	USA	AY010242	B	B	C						3
Okeechobee, FL	USA	AY010244	L	B	C	B	C				5
Mississippi	USA	AY010243	D	D	D	D	E				5
Missouri	USA	AY127052	B	B	B	B					4
Illinois	USA	AF345867	M	N	B	M	H				5
Texas	USA	AF428091	O	B	M	P					4

Figure 6: Structure of the MSP1a tandem repeats region using part of the tandem repeats sequences described in Figure 5. Source: DE LA FUENTE et al. (2007a).

The *msp1α* gene has been analyzed extensively to define variations and to characterize the genetic diversity of *A. marginale* strains worldwide (PALMER et al., 2001); (DE LA FUENTE et al., 2007a); (POHL et al., 2013); (CABEZAS-CRUZ et al., 2013a); (MUTSHEMBELE et al., 2014). Most tandem repeats are shared between different strains, and repeat B is the most common, occurring in 43 strains (CABEZAS-CRUZ et al., 2013a). The analysis of these sequences has not been able to provide phylogeographic information, as there is a weak association between the strains found and their places of origin (CABEZAS-CRUZ et al., 2013a). This configuration can be attributed to animal movement and other factors such as the convergent evolution of *A. marginale* strains (CABEZAS-CRUZ et al., 2013a); (CABEZAS-CRUZ et al., 2015).

Due to the variation in the repeating portion, the *msp1α* gene has been used as a stable genetic marker for identifying geographic isolates of *A. marginale* (BARBET et al., 1987); (BARBET et al., 2001); (DE LA FUENTE et al., 2001a); (DE LA FUENTE et al., 2001c). It is believed that the genetic diversity of *A. marginale* may occur during the sequential replication cycles that characterize persistent infection (KIESER et al., 1990), or during replication in the midgut and salivary glands of vector ticks (KOCAN, 1986); (KOCAN et al., 1993). This genetic diversity does not result in evolution, unless a more appropriate variant appears in

conditions that promote imbalance and favor rapid random evolution. (DE LA FUENTE et al., 2001c). Thus, wild-type or master sequences would remain unchanged (DE LA FUENTE et al., 1999). Selective forces can act on proteins that are under the effect (DE LA FUENTE et al., 1999) of structural or functional obstacles, resulting in convergent parallel evolution (homoplasy) (MASSEY et al., 2008). It is observed that tandem repeats evolve in a convergent manner towards a specific tandem repeat, probably due to a functional obstacle related to the role of this protein in pathogen–host interaction (CABEZAS-CRUZ et al., 2015).

The function of MSP1a as an adhesin can be influenced by both amino acid sequences and protein conformation (DE LA FUENTE et al., 2001a). The presence of negatively charged amino acids, such as aspartic acid (D) and glutamic acid (E) at position 20, have been proven essential for MSP1a binding to tick cell extract (TCE). In previous experiments, there was (at least in some strains) correlation between the presence of the cell extract ligand with tandem repeats transmissible by ticks of *Dermacentor* sp. (DE LA FUENTE et al., 2001a). Of 161 strains of *A. marginale*, at least 114 (71%) exhibited amino acids D or E at position 20, this position being the one with the most variability, suggesting that there is high evolutionary pressure on this amino acid position. (CABEZAS-CRUZ et al., 2013a).

CABEZAS-CRUZ et al. (2013a) observed that the 2D structure of the tandem repeats could also influence *A. marginale* transmission. Analysis revealed that amino acid 20 could be related to changes in the 2D structure of tandem repeats. Strains that were not transmitted by *Dermacentor* sp. predominantly had a 2D structure pattern different from those transmitted, regardless of the presence or absence of TCE–ligand tandem repeats (CABEZAS-CRUZ et al., 2013a). The relationship between the presence of TCE binders, the 2D structure of the tandem repeats, and the transmission by ticks is considered complex (RUIZ et al., 2005); (FUTSE et al., 2003);(BARBET et al., 1999). CABEZAS-CRUZ et al. (2013a) concluded that, for a strain to be transmitted by ticks, it must have a 2D structure that allows this transmission, or its tandem repeats must have TCE binders.

CABEZAS-CRUZ et al. (2013a) observed the importance of the first repeat (R1) in the interaction with ticks. However, high genetic variability would justify the existence of more than 20 different tick species considered to be transmitters of *A. marginale*, which made the authors consider the role of non-R1 sequences in the interaction with ticks. CABEZAS-CRUZ et al. (2013a) studied tandem repeats

separately by ecoregion according to rainfall and climatic characteristics. The results showed that there was greater variability of R1 sequence in areas where the vector species was *R.* (*Boophilus*) *microplus*. This confirms the hypothesis that the genetic diversity of *A. marginale* is associated with factors that promote the biological performance of vector ticks and their co-evolution with the arthropod (ESTRADA-PENA et al., 2009).

ESTRADA-PENA et al. (2009) also analyzed the microsatellite of *A. marginale* in ecoregions. They demonstrated that genotypes based on analysis of the *A. marginale* microsatellite are distributed differently in ecoregions; there is a significant difference in the distribution of these genotypes when comparing regions with high temperatures and medium/high rainfall with those with lower temperatures and rainfall.

MSP1a contains epitopes sensitive to B and T cell neutralization, which is necessary for the development of an immune response (DE LA FUENTE et al., 2001b); (BOWIE et al., 2002);(BROWN et al., 2001a; BROWN et al., 2001b; BROWN et al., 2002); (PALMER et al., 1987). MSP1a from *A. marginale* has been tested as an antigen candidate for bovine anaplasmosis (CABEZAS-CRUZ et al., 2015). Immunodominant epitopes have been found in these sequences (CABEZAS-CRUZ et al., 2015). Some of them have variation in these epitopes, showing a consequent evolution of negative pressure in some regions (MUTSHEMBELE et al., 2014); (CABEZAS-CRUZ et al., 2015). These changes are the consequence of mechanisms that allow the immunological escape of *A. marginale* by changing the recognition region in the tandem repeats of MSP1a, allowing immune response evasion at population level. (CABEZAS-CRUZ et al., 2015). A relevant observation is that, after acute infection, cattle are resistant when exposed to the same strain, but are fully susceptible to heterologous strains (KUTTLER, 1984); (CABEZAS-CRUZ et al., 2015). The biggest challenge in developing vaccine subunits for bovine anaplasmosis is the induction of cross-protection among strains that cause co-infection (CABEZAS-CRUZ et al., 2015).

The relationship between the high diversity observed in tandem repeats in the presence of biological vectors proposes that, together with vaccination based on the protein MSP1a, tick control should be done focusing on reducing the diversity of *msp1α* (CABEZAS-CRUZ et al., 2015). (CABEZAS-CRUZ et al., 2015) propose that developing vaccines using MSP1a as an antigen candidate should be based on

analysis of the composition of MSP1a and on genetic diversity. This would be possible by conducting local epidemiological studies and detecting the genetic variability of *A. marginale* based on the N-terminal region of MSP1a. From the results of these studies, it would be possible to develop a vaccine based on these peptides using common and conserved tandem replicates (CABEZAS-CRUZ et al., 2015).

2.3 Tick cell cultures

2.3.1 Use and properties of *in vitro* tick cell culture

Tick cell cultures present a simplified *in vitro* vector system that can be particularly useful for studies on intracellular and epicellular pathogens. Tick cell lines are particularly useful for studying tick-borne pathogens, including basic parasitic biology, host–vector–pathogen relationships, and disease control (BELL-SAKYI et al., 2007). Since their establishment approximately 50 years ago, tick cell lines have been used extensively to isolate, propagate, and study tick-borne viruses and bacteria and also in many tick–pathogen interaction studies (BELL-SAKYI et al., 2018). The study of agents in tick cell lines has great potential to provide information that cannot always be observed in *in vivo* studies, or in studies that would be expensive, in addition to the advantage of not requiring animal experimentation.

Most tick cell lines derive from embryonic cells (BELL-SAKYI et al., 2007); tick eggs are an easy-to-use stage of development and an abundant source of relatively undifferentiated material with (at least theoretically) rapid growth potential (BELL-SAKYI et al., 2018). The surfaces of engorged tick females are sterilized before oviposition begins. After oviposition, the egg surfaces are also sterilized. When embryo development is visible, the eggs are carefully broken up in balanced saline or culture medium to release the embryos, and the resulting tissue suspension is incubated in sealed containers between 28°C and 34°C (BELL-SAKYI et al., 2018). These cells usually do not require regular subculturing and can be kept as individual cultures with weekly medium replenishment for many months or years; all are relatively slow-growing, tolerate high densities, and grow in three dimensions

(BELL-SAKYI et al., 2018). Frozen Ixodidae cells can remain viable for many years in liquid nitrogen (BELL-SAKYI et al., 2018).

Many studies have used *in vitro* tick cells for isolating, multiplying, and characterizing tick-borne intracellular bacteria (BELL-SAKYI et al., 2018). The ability of these cells to survive for many months without subculturing facilitates the difficult isolation of very slow-growing bacterial species, which can be present in extremely small amounts in the inoculum (BELL-SAKYI et al., 2018). There are currently a large number of *in vitro*-isolated tick-transmitted intracellular bacteria (BELL-SAKYI et al., 2018). These bacteria include organisms of the genera *Anaplasma*, *Ehrlichia*, *Rickettsia*, and *Spiroplasma* (BEKKER et al., 2005; SHKAP et al., 2008; WOLDEHIWET, 2010; ALBERDI et al., 2012; ZWEYGARTH et al., 2014a; CABEZAS-CRUZ et al., 2016; ABDELKADIR et al., 2019).

2.3.2 *Anaplasma marginale* in tick cell culture

The first attempts to propagate *A. marginale* were limited to the erythrocytic phase, using a blood culture system based on a method used for *Plasmodium* (KESSLER et al., 1979), and did not result in continuous spread (PASSOS, 2012). Another attempt to cultivate *A. marginale* was based on the use of mosquito-derived cells, but there was also no spread (MAZZOLA et al., 1976). MUNDERLOH et al. (1996) performed the first continuous *in vitro* cultivation of *A. marginale* in *I. scapularis* cells (IDE8). Currently, isolation is based on the methodologies described by MUNDERLOH et al. (1996) and BLOUIN et al. (2000). The principle of the process is based on cultivating *A. marginale* started from blood obtained from infected calves during peak rickettsemia (BLOUIN et al., 2000); (BASTOS et al., 2009) and also from chronically infected calves (BAETA et al., 2015).

BLOUIN et al. (1998) followed the infection process after IDE8 cells had been exposed to *A. marginale*. Invasion of the host cell starts within 15 minutes with the dense form of *A. marginale* (0.5–0.8 µm) adhering to the host cell membrane. The adhesion between rickettsia and the host cell intensifies over time, forming a depression in the host cell. Then, *A. marginale* is internalized, thus creating a parasitophorous vacuole. Inside the vacuole, the bacteria take on the reticulated (vegetative) form, which divides by binary fission. Divisions occur continuously and

result in the formation of colonies containing hundreds of organisms. Subsequently, the reticulated forms become dense forms, which are rounded and contain a dense and more uniform distribution of ribosomes, in which the DNA fibrils are not apparent. Then, the membranes of the colonies fuse to the host cell plasmalemma, followed by rupture of the membrane complex. A flap opens in the fused cell membranes and allows the dense forms to be released from the parasitophorous vacuole without loss of host cell cytoplasm. The free bacteria initiate a new series of infections, resulting in host cells that can contain five or more colonies per cell (BLOUIN et al., 1998). Cell death *in vitro* occurs after most cells become infected, resulting in shedding of the cell monolayer, with apparent cytopathic effect when observed under light microscopy (BLOUIN et al., 2002).

As *A. marginale* was isolated in IDE8 cells, this cell line has been used for propagating *A. marginale* isolates from several countries such as the USA (MUNDERLOH et al., 1996); (KOCAN, 1986); (BLOUIN et al., 2000), South Africa (ZWEYGARTH et al., 2006b), and Brazil (BASTOS et al., 2009); (BAETA et al., 2015).

Anaplasma marginale grown in tick cells are infective for cattle and are potential antigens for use in vaccine preparations and serological tests (BELL-SAKYI et al., 2007). The use of vaccines derived from cell cultures would be convenient, as production is easily standardized, free from the stroma of bovine erythrocytes and possible contaminating pathogens, and eliminates the need to use cattle for producing antigens (PASSOS, 2012).

Having local strains *in vitro* allows not only knowledge of their pathogenicity, morphology, and biology, but also grants access to the agent whenever necessary to obtain immunogenic material or material of any other nature for more studies.

2.4 Genus *Anaplasma*

The main species assigned to the *Anaplasma* genus are *A. marginale*, *A. centrale*, *A. bovis*, *A. ovis*, *A. platys*, and *A. phagocytophilum*, according to the reclassification proposed by DUMLER et al. (2001) based on the 16S rRNA and groELS genes.

This genus is composed of gram-negative organisms, often pleomorphic, coccoid to ellipsoidal that reside inside cytoplasmic vacuoles, individually or more often in compact inclusions called morulae, present in mature and immature hematopoietic cells, particularly the myeloid, neutrophil, and erythrocyte cells, in peripheral blood or tissues, usually in the phagocytic mononuclear organs (spleen, liver, and bone marrow) of mammalian hosts (DUMLER et al., 2001).

Anaplasma spp. are important agents associated with ticks for veterinary medicine and public health; they cause anaplasmosis in several animal species and in humans (ATIF, 2016).

2.4.1 *Anaplasma phagocytophilum*

DUMLER et al. (2001) proposed replacing the names of three species, *Ehrlichia phagocytophila*, *E. equi*, and human granulocytic ehrlichiosis agent with *A. phagocytophilum* after the reorganization of the *Rickettsiaceae* and Anaplasmataceae families in the Rickettsiales order. *Anaplasma phagocytophilum* is widespread in Europe, the USA, and Asia; it was discovered in South America and Africa (BATTILANI et al., 2017). It infects neutrophils and eosinophils, forming colonies called morulae (WOLDEHIWET, 2010). In the later stages of bacteremia, they are also found in monocytes (WOLDEHIWET et al., 1982; WOLDEHIWET, 1987).

The percentage of infected phagocytic cells depends on the stage of bacteremia, host susceptibility, and the bacterial strain involved (BATTILANI et al., 2017). Infected patients experience immunosuppression and are subject to opportunistic infections (WOLDEHIWET, 2010). Symptomatic cases are of varying severity, and may range from mild, with self-limiting fever, to lethal cases (DUMLER et al., 2005). Hospitalizations have been observed in half of the patients with advanced age, high neutrophil count, low lymphocyte count, anemia, presence of morulae in leukocytes, or underlying immunosuppression (BAKKEN et al., 1996). In the case of infection in farm animals, the disease caused by *A. phagocytophilum* can be subclinical or have severe symptoms such as fever, drowsiness, anorexia, abortions, decreased body weight, and reduced milk production. Patients who are

treated late, especially weaker individuals, may die (STUEN et al., 2002; STUEN, 2007).

The tick complex *I. persulcatus* includes *I. ricinus* in Europe; *I. scapularis*, *I. pacificus* and *I. spinipalpis* in the USA; and *I. persulcatus* in Asia and Russia. These ticks are vectors of *A. phagocytophilum* (WOLDEHIWET, 2010). The tick infection is established after a blood meal, and the bacteria are transmitted via the transstadial, but not the transovarian pathway (DUMLER et al., 2001).

2.4.2 *Anaplasma bovis*

Anaplasma bovis is a bacterium mainly found in cattle and buffaloes, and can also be found in goats, dogs, deer, and other hosts (UILENBERG, 1993; ATIF, 2016). It infects monocytes and is frequently detected in cattle, goats, and deer (LIU et al., 2012; CECI et al., 2014; YANG et al., 2018). It has been identified in other animal species such as dogs, rabbits, and cats, and in small mammals (GOETHERT et al., 2003; YOSHIMOTO et al., 2010; SASAKI et al., 2012; MASUZAWA et al., 2014). It has been reported in Brazil, North America, Africa, Japan, and Europe (GOETHERT et al., 2003; KAWAHARA et al., 2006; SANTOS et al., 2006; RAR et al., 2011). *Anaplasma bovis* infection causes fever, decreased milk production, weakness, weight loss, and mucosal pallor, and in stressed cattle, it can lead to death (LOSOS, 1986; SREEKUMAR et al., 1999).

The tick species suspected of being involved in *A. bovis* transmission are *Hyalomma* spp., *Amblyomma variegatum*, *R. appendiculatus*, *R. sanguineus*, and *Haemaphysalis* spp. (UILENBERG, 1993; DUMLER et al., 2001; GOETHERT et al., 2003; HARRUS et al., 2011; PALOMAR et al., 2015).

2.4.3 *Anaplasma centrale*

Anaplasma centrale is an intra-erythrocytic bacterium that is closely related to *A. marginale*, although it forms smaller and more central inclusions inside infected blood cells (POTGIETER et al., 1987). It is distributed in tropical and subtropical regions (RAR et al., 2011) and infects cattle and other ruminants such as sheep,

wildebeests, antelopes, and deer (HOSSEINI-VASOUKOLAEI et al., 2014; LI et al., 2015a) (KHUMALO et al., 2018). *Anaplasma centrale* infection causes mild clinical symptoms and confers long-term protective immunity against highly virulent strains of *A. marginale* (KOCAN et al., 2003; RAR et al., 2011). For this reason, this species is used as a live vaccine against *A. marginale* (KOCAN et al., 2010).

2.4.4 *Anaplasma ovis*

Anaplasma ovis infects small ruminants such as goats and sheep, and its presence has been confirmed in most regions of the world, in both farm animals and wildlife (KUTTLER, 1984). Similar to *A. marginale* and *A. centrale*, this pathogen is found inside erythrocytes, causing anemia in infected animals (SPLITTER et al., 1956).

Anaplasma ovis transmission occurs biologically via species of hard and soft ticks including *R. bursa*, *D. andersoni*, *R. turanicus*, *Ornithodoros lahorensis*, and mechanically by diptera bites (ATIF, 2016).

Animals infected with *A. ovis* usually show the mild form of the disease (BATTILANI et al., 2017). Acute infection is characterized by fever, weakness, anemia, pallor of the mucous membranes, decreased milk production, jaundice, abortions, and often death (SHOMPOLE et al., 1989; KOCAN et al., 2003).

2.4.5 *Anaplasma platys*

Anaplasma platys was classified after the reorganization of the family Anaplasmataceae by DUMLER et al. (2001); it was previously called *E. platys*. This pathogen is an obligate parasite of platelets, and has been detected on all continents (HARVEY et al., 1978; SANOGO et al., 2003; MARTIN et al., 2005; AGUIRRE et al., 2006; MELO et al., 2016). It is known as a dog pathogen, but infection has also been reported in cats, foxes, camels, deer, cattle, and humans (HARVEY et al., 1978; MAGGI et al., 2013; QUROLLO et al., 2014; CARDOSO et al., 2015; DAHMANI et al., 2015; LI et al., 2015a; LI et al., 2015b).

In dogs, the clinical signs are characterized by lethargy, anorexia, fever, purulent eye discharge, splenomegaly, and petechial hemorrhage (SAINZ et al., 1999; TROTTA et al., 2009). Laboratory findings include thrombocytopenia, anemia, macroplatelets, and monocytosis (HARRUS et al., 1997; DAGNONE et al., 2003; ANTOGNONI et al., 2014). Asymptomatic infection can occur in animals as well (DE CAPRARIIS et al., 2011).

The involvement of *R. sanguineus* in its transmission is suspected (INOKUMA et al., 2000; SANOGO et al., 2003). *Anaplasma platys* has been found in *R. sanguineus* adults and nymphs from negative dogs, suggesting the occurrence of transstadial transmission (RAMOS et al., 2014).

2.4.6 Emerging species within the genus *Anaplasma*

Recent studies have shown the presence of other organisms of the genus *Anaplasma* in vertebrates and invertebrates. Ruminants appear to be the key in the epidemiology of the genus *Anaplasma*, acting as both host and reservoir of infection (ZOBBA et al., 2014). All bacterial species of the genus *Anaplasma* infect wild and domestic ruminants worldwide (ZOBBA et al., 2014; DAHMANI et al., 2015).

In China, a bacterium called *A. capra* has been described in humans with a history of tick bites (LI et al., 2015a). This microorganism is considered important for public health, because it causes clinical disease in humans, which is characterized by fever, malaise, headache, dizziness, and chills (LI et al., 2015a). The same agent has also been found in the ticks *I. persulcatus*, *Haemaphysalis longicornis*, and *Haemaphysalis qinghaiensis*, as well as in ruminants such as goats, sheep, and deer (SATO et al., 2009; LI et al., 2014; LI et al., 2015a; LI et al., 2015b; LI et al., 2015c; YANG et al., 2016; PENG et al., 2018).

Another organism of the genus *Anaplasma* was detected in Italy by ZOBBA et al. (2014) in cattle. This bacterium showed 99% identity with the 16S rRNA gene sequence of *A. platys* from dogs, and was designated *A. platys*-like. In phylogenetic analysis, this microorganism was grouped into a distinct subcluster closely related to a subcluster that consists of *A. phagocytophilum* and *A. bovis* (ZOBBA et al., 2014).

This bacterium also shows tropism to neutrophils and not to platelets (ZOBBA et al., 2014).

In Morocco and Saudi Arabia, an agent termed *Candidatus Anaplasma camelii* has been described in camels (BASTOS et al., 2015; AIT LBACHA et al., 2017). The groEL gene of this organism is very similar to that of *A. platys*. Some of the positive animals showed clinical symptoms (AIT LBACHA et al., 2017).

Another microorganism also has high similarity with *A. platys* after analysis of the 16S rRNA, GroELS, gltA, and rpoB genes (TATE et al., 2013). This rickettsia, identified in white-tailed deer in the USA, is called *A. odocoilei*. Electron microscopy, purified platelet PCR, immunohistochemistry and *in situ* hybridization have confirmed platelet infection (TATE et al., 2013). Similar to dogs infected with *A. platys* HARVEY et al. (1978), infected deer showed no clinical signs, although there were 2–4 episodes of thrombocytopenia (TATE et al., 2013). *Anaplasma odocoilei* has been isolated *in vitro* in tick cells, which made it possible to use material to analyze its identity and biology (TATE et al., 2013). This agent can be expanded and cryopreserved for future experiments, some of which can even omit animal studies (BELL-SAKYI et al., 2007; BELL-SAKYI et al., 2018).

CHAPTER I

DIVERSITY ANALYSIS OF THE *Anaplasma marginale msp*1α GENE IN CALVES AND TICKS AND ITS RE-ISOLATION FROM THE BUFFY COAT

ABSTRACT

SANTOS, Priscilla Nunes dos. **Diversity analysis of the *Anaplasma marginale msp*1α gene in calves and ticks and its re-isolation from the buffy coat.** 2020. 29p. Thesis (PhD in Veterinary Sciences). Veterinary Institute, Department of Epidemiology and Public Health, Federal Rural University of Rio de Janeiro, Seropédica, RJ, 2020.

Anaplasma marginale is an agent transmitted by ticks and is responsible for bovine anaplasmosis. Its major surface protein MSP1a is encoded by the *msp*1α gene and has proven to be a valuable target in diversity studies, as it is extremely important for its evidence in relation to co-evolution with *A. marginale* tick vectors. Bacterial replication is considered to occur only in erythrocytes, when infecting ruminants. This study aimed at evaluating *A. marginale* MSP1a protein structure diversity in experimentally infected bovines and ticks, and re-isolating, from the buffy coat, the strain used in the infection. The experimental study was conducted for 1 year. Two calves were infested with 1 g *Rhipicephalus microplus* ticks and inoculated intravenously, each receiving 1.5 mL culture suspension containing a different *A. marginale* strain. The two calves were kept in a stall and observed daily via blood smears, rectal body temperature, hematocrit, total plasmatic proteins, and PCR of blood samples until day 45 after inoculation. After that, the animals were removed from the stall and kept in paddocks, and the same parameters were observed monthly. One hundred ticks were collected from each animal during the stall period. During the pasture period, 61 ticks were harvested and subsequently dissected. DNA was extracted from blood samples and tick organs and underwent PCR for the *msp*5 and *msp*1α genes. The *msp*1α gene was sequenced. Chronic infection was observed after inoculation. The animals showed no changes in the parameters evaluated due to anaplasmosis. There was no variation in MSP1a in either of the two experimental animals, nor in the positive ticks for the *msp*1α gene. The permanence of the MSP1a structure reveals the low frequency of variation occurring in this protein. *Anaplasma marginale* was re-isolated from the buffy coat; the first culture was performed 140 days after infection, another two cultures at 143 days, and one culture at 170 days after the inoculation. Two of the cultures were submitted to PCR and sequencing for the *msp*1α gene and were compatible with the AmRio1 sequences. The isolation of

A. marginale from the buffy coat presents the possibility that its life cycle is associated with other cells than erythrocytes.
Keywords: AmRio1; MSP1a; buffy coat; *msp*1α

RESUMO

SANTOS, Priscilla Nunes dos. **Análise da diversidade do gene *msp*1α de *Anaplasma marginale* em bezerros e carrapatos e seu re-isolamento a partir da capa leucocitária**. 2020. 29p. Tese (Doutorado em Ciências Veterinárias). Instituto de Veterinária, Departamento de Epidemiologia e Saúde Pública, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2020.

Anaplasma marginale é um agente transmitido por carrapatos e é responsável pela anaplasmose bovina. Sua proteína principal de superfície MSP1a é codificada pelo gene *msp*1α e provou ser um alvo valioso em estudos de diversidade, pois é extremamente importante por suas evidências em relação à co-evolução com carrapatos vetores de *A. marginale*. Considera-se que ao infectar ruminantes, a replicação bacteriana ocorre apenas nos eritrócitos. Este estudo teve como objetivo avaliar a diversidade da estrutura da proteína MSP1a de *A. marginale* em bovinos e carrapatos infectados experimentalmente e re-isolar, a partir capa leucocitária, uma estirpe usada na infecção. O estudo experimental foi realizado por 1 ano. Dois bezerros foram infestados com 1 g de carrapatos *Rhipicephalus microplus* e inoculados por via intravenosa, cada um recebendo 1,5 mL de suspensão de cultura contendo uma estirpe de *A. marginale* diferente. Os dois bezerros foram mantidos estabulados e observados diariamente via esfregaços de sangue, temperatura corporal, hematócrito, proteínas plasmáticas totais e PCR de amostras de sangue até o dia 45 após a inoculação. Em seguida, os animais foram retirados das baias e mantidos em piquetes, e os mesmos parâmetros foram observados mensalmente. Cem carrapatos foram coletados de cada animal durante o período de confinamento. Durante o período a pasto, 61 carrapatos foram colhidos e posteriormente dissecados. O DNA foi extraído de amostras de sangue e órgãos dos carrapatos e submetido à PCR para os genes *msp*5 e *msp*1α. O gene *msp*1α foi sequenciado. Foi observada a infecção crônica após a inoculação. Os animais não apresentaram alterações nos parâmetros avaliados devido à anaplasmose. Não houve variação em MSP1a em nenhum dos dois animais experimentais, nem nos carrapatos positivos para o gene *msp*1α. A estabilidade da estrutura MSP1a revela a baixa frequência de variação que ocorre nesta proteína. *Anaplasma marginale* foi re-isolado da capa leucocitária; a primeira cultura foi realizada 141 dias após a infecção, outras duas culturas aos 143 dias e uma cultura aos 172 dias após a inoculação. Duas das culturas foram submetidas a PCR e sequenciamento

para o gene *msp*1α e eram compatíveis com as sequências de MSP1a da estirpe AmRio1. O isolamento de *A. marginale* da capa leucocitária revela a possibilidade de que seu ciclo de vida esteja associado a outras células e não somente aos eritrócitos.
Palavras-chave: AmRio1; MSP1a; capa leucocitária; *msp*1α

1 INTRODUCTION

Bovine anaplasmosis is a disease caused by the bacterium *Anaplasma marginale*, which is transmitted by ticks to cattle and wild ruminants (KOCAN et al., 2010). The disease occurs worldwide in tropical and subtropical areas, including North and South America, Africa, the Caribbean, Russia, European countries neighboring the Mediterranean, the Middle East, and the Far East (KOCAN et al., 2010). It is transmitted biologically by ticks, mechanically by fomites or the mouthparts of blood-sucking flies, and less commonly by transplacental transmission (AUBRY et al., 2011).

Many *A. marginale* strains have been identified, differing in biology, genetic characteristics, and tick transmissibility (DE LA FUENTE et al., 2001c; DE LA FUENTE et al., 2005). Major surface proteins (MSPs) have been used to characterize *A. marginale* strains genetically (DE LA FUENTE et al., 2005). One of the most important MPSs is MSP1a, encoded by the *msp*1α gene and identified only in the *A. marginale* species (DE LA FUENTE et al., 2005). MSP1a is considered an adhesin for bovine erythrocytes and tick cells (MCGAREY et al., 1994a; MCGAREY et al., 1994b; DE LA FUENTE et al., 2001b) in addition to having epitopes for B cells (CABEZAS-CRUZ et al., 2013b). Its molecular weight varies between geographical isolates because of the variation in the number of tandem repeats and amino acids (23–31 amino acids) in its N-terminal portion (ALLRED et al., 1990; DE LA FUENTE et al., 2005; DE LA FUENTE et al., 2007b; CABEZAS-CRUZ et al., 2013b). Analysis of the tandem repeats of MSP1a has contributed to understanding of the genetic diversity of *A. marginale* within specific regions, and has yielded information on the evolution of the pathogen–host interaction (DE LA FUENTE et al., 2003b; DE LA FUENTE et al., 2004b; DE LA FUENTE et al., 2007b; ESTRADA-PENA et al., 2009).

It is possible that the genetic diversity of *A. marginale* occurs during the sequential replication cycles that characterize persistent infection (KIESER et al., 1990) or during replication in the midgut and salivary glands of its tick vectors (KOCAN, 1986; KOCAN et al., 1993). Studies evaluating the genetic diversity of *A. marginale* in cattle and ticks individually were conducted in climatic conditions different from those found in Brazil, and the evaluation was conducted in ticks of the genus *Dermacentor* (Palmer et al., 2001). ESTRADA-PENA et al. (2009) observed greater

diversity in ecoregions with climatic conditions compatible with that of Brazil, and suggested the involvement of the genus *Rhipicephalus* spp. in the evolutionary process of *A. marginale* strains.

MUNDERLOH et al. (1996) were the first to propagate *A. marginale* in an *Ixodes scapularis* cell culture (IDE8 cells). Bovine erythrocytes are used to inoculate IDE8 cells; these cells have been considered the only replication site in vertebrate hosts (BLOUIN et al., 2002; MUNDERLOH et al., 2004). By attaching to the host, pathogens are inoculated via tick saliva and a lesion forms around the hypostome (NUTTALL, 1998; ANDERSON, 2002). The bite location is an inflammatory focus that attracts the first line of response of inflammation, such as neutrophilic granulocytes (TATCHELL et al., 1970), changing their behavior (BORJESSON et al., 2003). (MUNDERLOH et al., 2004) cultivated *A. marginale* in endothelial cell lines, proposing that the agent could also be able to infect nucleated cells.

Therefore, the present study was aimed at evaluating the diversity of the MSP1a tandem repeat structure of *A. marginale* in experimentally infected cattle and in *R. microplus* over a 1-year period. The *A. marginale* strain AmRio1 (BAETA et al., 2015) was re-isolated from the buffy coat.

2 MATERIAL AND METHODS

2.1 Experimental animals

Two calves were used in this study; both were crossbred and from the Agricultural Research Corporation of Rio de Janeiro (PESAGRO-RIO), and were post-colostrum ingestion and not splenectomized. Calf 1 was 17 days old and Calf 2 was 8 days old. The animals were kept in one stall each in a screened environment to prevent dipteran entry. Both calves were fed a 1:2 ratio of cow's milk diluted in artificial milk substitute during the first 4 days. Then, the proportion of cow's milk was reduced to 1:3 for 2 days. From then on, the animals were fed only with the artificial milk substitute for 36 days. Over these 36 days, feed (Terneirina®, Purina) and alfalfa hay were introduced gradually.

The animal tick treatments were performed using 12.5% amitraz (Triatox®, MSD, Brazil). The acaricide was applied by spraying according to the manufacturer's recommendations for 3 consecutive days. Possible ticks were eliminated with a blowtorch from the environment in the animals' housing.

Five days after the animals' arrival, a blood sample was taken to make blood smear slides and the *msp*5 gene PCR (SINGH et al., 2012) for detecting *A. marginale*. No morphologically compatible structure to *A. marginale* was observed under light microscopy. The *msp*5 gene PCR showed that the animals were negative for the agent.

After the weaning period, which corresponded to 28 days after the animals' first blood evaluation, an evaluation was carried out using a new blood collection. On this date, the erythrocytes in the animals' blood smears had inclusions suggestive of *A. marginale*. PCR for the *msp*5 gene was positive.

To eliminate *A. marginale* from the animals, oxytetracycline (Terramicina® LA, Zoetis, Brazil) at 22 mg/kg was administered intravenously for 5 consecutive days as recommended by the Office International des Epizooties (OIE, World Organization for Animal Health).

After treatment and the end of the withdrawal period, the calves were evaluated again. There were no inclusions in the blood smears and both calves were negative for the *A. marginale msp*5 and *msp*1α genes (LEW et al., 2002); thus, they were considered suitable for experimental infection.

The experimental infection was carried out using two strains of *A. marginale* from cell culture. Animal 1 received the AmRio1 strain and Animal 2 received the AmRio2 strain. Both strains were previously isolated by (BAETA et al., 2015). The inocula were administered in a volume of 1.5 mL in the jugular vein of each animal.

After the experimental infection, the animals were kept in a stall to monitor clinical signs in case of acute infection. After 45 days, the animals were released in a 6-hectare *Brachiaria humidicola* and *B. decumbens* pasture. The animals were evaluated monthly over the course of 1 year to monitor persistent infection.

This project was submitted to the ethics committee on the use of animals (CEUA) and approved with protocol number 2134171215.

2.2 Infestation of animals with *Rhipicephalus microplus* ticks

Ten days before the experimental infection, the animals were infested in a stall with 1 g larvae (20,000 larvae) of *Rhipicephalus microplus* ticks (VARGAS et al., 2003) 15 days after hatching. The colony was maintained in the Parasitic Diseases Laboratory of the Federal Rural University of Rio de Janeiro (UFRRJ). The ticks used belonged to the Porto Alegre strain, were pathogen-free, and kept on Holstein calves (*Bos taurus*) (RECK et al., 2009). The colony was kept in a chamber at 27°C, with 80% relative humidity.

2.3 Maintenance of IDE8 cells and AmRio1 and AmRio2 strains

The cell line used was the IDE8 cell line of embryonic cells of *I. scapularis* (MUNDERLOH et al., 1994), which were kept at 28°C in 12.5-cm² flasks in Leibovitz's L-15B medium supplemented with 5% fetal bovine serum (Sigma-Aldrich®, USA), 10% tryptase phosphate broth, 0.1% concentrated bovine lipoprotein (MP Biomedicals®), 1% L-glutamine (200 mM), and 1% solution with the antibiotics penicillin (10,000 IU) and streptomycin (10 mg/mL). The medium pH was adjusted to 6.6. The medium was replaced weekly and the cells were passaged when monolayer confluence was observed. The cells were monitored using an inverted light microscope.

The strains AmRio1 and AmRio2 (BAETA et al., 2015) were kept at the UFRRJ Laboratory of Parasitic Diseases in the IDE8 embryonic cell line. *Anaplasma*

marginale strains were maintained in L15B medium with 5% fetal bovine serum, $NaHCO_3$ (2.5 g/L), and HEPES (MUNDERLOH et al., 1996). The medium was replaced twice per week and the agent was subcultured approximately every 30 days.

2.4 Inoculum preparation

The solution inoculated in the animals to carry out the infection was prepared from IDE8 cell culture presenting a percentage of *A. marginale* infection of >70%. The infection causes the cells to detach from the surface of the flask. Possible remaining cells still adhered to the flask wall were removed by rinsing with the culture medium contained in the flask. The cells were ruptured by passing them through a curved 25 × 7 mm needle attached to a syringe. This procedure was performed for both strains. After disruption, the medium containing the ruptured cells in a sterile beaker was transferred to a syringe for inoculation.

The inoculation was performed through the jugular vein, with no volume of 1.5 mL, using a 25 x 7 mm needle, 10 days after the infestation of the animals.

2.5 Clinical and hematological monitoring of experimental animals

Immediately after the experimental infection, the animals were kept in a stall; they underwent daily temperature measurements, blood collection in tubes containing EDTA (ethylene diamine tetra-acetic acid), and blood smear preparation and hematocrit and total plasmatic protein (TPP) measurement.

Blood was collected using 5 mL EDTA tubes (BD®) and 8 × 30 mm needles via puncture of the jugular vein.

Twenty-eight days after the first attempt, a new infection with the AmRio2 strain was performed on Animal 2. The infection was carried out using the same methodology used in the first cultured material inoculation. It has been continued to obtain samples from both animals. In Animal 1, samples were obtained daily until day 42 after inoculation. In Animal 2, samples were drawn every day until day 45 after the first inoculation and until day 20 after the second inoculation. Forty-five days after inoculation, the animals were released to pasture.

When the animals were loose in the pasture, samples were collected monthly. Temperature, TPP, hematocrit, and blood smears were also evaluated during this

period. The evaluation of these parameters during the pasture period ended in the month corresponding to that of inoculation, 1 year later.
During the follow-up period, blood samples were collected for re-isolating *A. marginale* from Animal 1 in IDE8 cells culture.

2.6 Blood sample processing

2.6.1 Blood smears

After blood smears had been prepared (from capillary and venous blood), they were fixed in P.A. methanol for 3 minutes, stained for 45 minutes in Giemsa (Sigma-Aldrich®), diluted in 10% distilled water. Subsequently, the smears were observed under optical microscopy (Olympus BX41®, Japan) at 1000× magnification.

2.6.2 Hematocrit

To estimate the mean corpuscular volume (MCV), microhematocrit tubes filled with blood up to 2/3 of their length were used. The tubes were sealed at one end with a mass suitable for sealing microhematocrit tubes and were centrifuged at 4500 rpm (Fanem®, Brazil) for 5 minutes. Duplicate tubes were prepared for each sample tested. A reading ruler was used to interpret microhematocrit (Fanem®, Brazil) and to determine the percentage of red blood cells in relation to the total blood volume from the average result of the two microhematocrit tubes (DOUGLAS et al., 2010).

2.6.3 TPP

The microhematocrit tubes were broken off at the portion that contained only plasma. The plasma was deposited in a refractometer (Kasvi, Brazil) to determine the TPP concentration.

2.7 Obtaining samples from ticks

Seventy semi-engorged females destined for dissection were obtained from each animal 19 days after infestation (11 days after infection), and 30 semi-engorged females were collected 40 days after infestation (32 days after infection) from the animals for dissection.

The females were dissected according to Sá et al (2018) to remove the salivary glands and guts. In a petri dish, the females were dissected through the anterior portion and the shells were depleted in phosphate-buffered saline (PBS, pH 7.4) to release the organs into the buffer at room temperature. The organs were placed in polypropylene tubes with sufficient volume of RNAlater Stabilization Solution (Thermo Fisher Scientific®, USA) to immerse the organs.

After dissection and obtaining 100 tick gut samples and tick salivary glands from both animals, the animals were treated with amitraz (Triatox®, MSD) for 3 consecutive days and kept in a stall for 1 week, which had been treated with a blowtorch to eliminate all ticks.

During the follow-up period of persistent infection, the animals had natural infestation by grazing ticks. The animals were evaluated monthly, and semi-engorged females were removed from their bodies and dissected. A total of 61 ticks were collected from each animal for further dissection during this period. In both animals, ticks were harvested on day 104, 147, 167, 209, 272, 307, 336, and 367 after inoculation (considering the first inoculation as a parameter in animal 2). The ticks were stored in polypropylene tubes in a sufficient volume of RNAlater solution for immersing the organs in a -20°C freezer.

2.8 *Anaplasma marginale* re-isolation

The AmRio1 strain was re-isolated according to the protocol proposed by (ZWEYGARTH et al., 2014b) for isolating *E. canis* in monocytes. Four re-isolation attempts were made. The first attempt was made 140 days after inoculation of the agent. The second and third attempts were made 143 days after infection. The fourth attempt was made 170 days after the experimental infection.

Blood was collected from the jugular vein of Animal 1 using a vacutainer system in an EDTA-containing tube. The blood was manipulated inside a laminar flow chamber to remove an aliquot to perform blood smears. The remaining blood (approximately 4 mL) was processed using Histopaque-1083® (Sigma-Aldrich®)

for separating the monocytes from whole blood. The whole blood was centrifuged in a sterile conical 15-mL polypropylene tube (Kasvi®) at 500 ×*g* for 10 minutes at room temperature. After centrifugation, the buffy coat was removed using a sterile pasteur pipette (Olen®, Brazil) and placed in a new conical tube. The buffy coat and contaminating erythrocytes were washed once in sterile PBS (pH 7.4) by adding 10 mL PBS and centrifuging the cells at 500 ×*g* for 10 minutes at room temperature. The supernatant was discarded and the cells were resuspended in 3 mL sterile PBS (pH 7.4). Histopaque-1083 (3 mL) was added slowly to a mixture of sterile PBS + white cells + contaminating erythrocytes in a volume of 3 mL. The mixture of buffer and blood cells occupied the upper portion of the tube while the Histopaque occupied the lower portion. The tube was centrifuged at 800 ×*g* for 20 minutes at room temperature. Then, the cells in the interphase were removed and placed in a new conical tube. These cells were washed twice using PBS (pH 7.4) in a volume of 10 mL and were centrifuged at 500 ×*g* for 10 minutes. After that, the cells were resuspended in L15B medium with 5% fetal bovine serum, $NaHCO_3$ (2.5 g/L), and HEPES (MUNDERLOH et al., 1996), and inserted in a 12.5 cm² flask (Kasvi®) containing uninfected IDE8 cells. After the IDE8 cells had been exposed for 24 hours to the white blood cells, the culture medium in the flask was completely replaced.

The blood samples taken 140, 143 and 170 days after inoculation were used for DNA extraction and subsequently tested for the *A. bovis* 16S rRNA gene and the *A. phagocytophilum msp*2 gene.

2.9 Culture monitoring

The infected IDE8 cells were maintained at 37°C, with 50% culture medium replacement twice a week. The cells were observed daily macroscopically and microscopically using an inverted light microscope (Nikon®, Japan) and every two weeks by observing cytocentrifuge smears under a light microscope (Olympus BX41®). Smears were observed at 1000× magnification.

Cytocentrifuge smears (Fanem®) were made from 0.5 mL culture. The culture was centrifuged at 1000 rpm for 10 minutes. The smears were fixed in P.A. methanol for 3 minutes and stained for 30 minutes in 10% Giemsa diluted in distilled water (Sigma-Aldrich®).

Semi-nested PCR were also performed for the *msp*5 and *msp*1α genes and sample sequencing from culture. The culture samples for DNA extraction were obtained 32 and 50 days after inoculation of the agent in IDE8 cells. PCR was performed for the *msp*5 and *msp*1α genes using the first extracted DNA, before the passage of the agent. The first extracted DNA was also used for PCR of the *A. bovis* 16S rRNA, Anaplasmataceae 16S rRNA and *A. phagocytophilum msp*2 genes as targets. Subsequently, a new extraction was performed after at least three passages, and the *msp*1α gene product from this DNA was sequenced.

2.10 DNA extraction

2.10.1 Blood and cell culture samples

DNA was extracted from the blood samples and cell culture using a commercial kit (Promega®, USA) according to the manufacturer's recommendations for blood extraction.

2.10.2 Tick organ samples

The phenol-chloroform method (Santolin, 2014) was used for extracting DNA from the tick salivary glands and guts. The tick organs were digested at 56°C overnight in a polypropylene tube containing 20 µL proteinase K (Sigma-Aldrich®) and 200 µL Digest Buffer (Santolin, 2014). The digested tick organs were centrifuged at 16,000 ×*g* for 1 minute. The supernatant (400 µL) from each sample was transferred to a new tube containing 200 µL HBSS (Hanks balanced salt solution, Sigma-Aldrich®) and 600 µL phenol in the fume hood, and the tube was inverted five times. The tube was centrifuged for 5 minutes at 16,000 ×*g*, and 550 µL supernatant was aliquoted into a new tube. Phenol-chloroform (550 µL) was added in the fume hood, and the tube was inverted five times. The mixture was centrifuged at 16,000 ×*g* for 5 minutes, and then 550 µL supernatant was aliquoted into a new tube. Isopropanol (500 µL) was added, and the tube was incubated at room temperature for 20 minutes before it was centrifuged for 20 minutes at 16,000 ×*g*. Then, the supernatant was discarded

and 500 µL 70% ice-cold ethanol was added, followed by centrifugation at 16,000 ×*g* for 5 minutes, and the supernatant was discarded. This last step was repeated under the same centrifugation conditions. The pellet resulting from the extraction was resuspended in 50 µL ultra-pure water and the extracted DNA was held at 8°C overnight. Subsequently, the samples were incubated at 56°C for 15 minutes, homogenized, and stored at -20°C.

2.11 Polymerase chain reaction (PCR)

2.11.1 The *msp*5 gene

The *msp*5 gene was detected in DNA samples using a semi-nested PCR as a screening for *A. marginale* detection using the following primers (SINGH et al., 2012):

Amar msp5 eF: 5′-GCATAGCCTCCGCGTCTTTC-3′ **(semi-nested PCR first reaction)**

Amar msp5 eR: 5′-TCCTCGCCTTGGCCCTCAGA-3′ **(semi-nested PCR first and second reactions)**

Amar msp5 iF: 5′-TACACGTGCCCTACCGAGTTA-3′ **(semi-nested PCR second reaction)**

In both reactions, the PCR conditions were: initial denaturation at 94°C for 5 minutes, followed by 34 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute. The final extension was at 72°C for 10 minutes.

A master mix was prepared with 1× final buffer, 1.5 mM $MgCl_2$, and 0.2 mM deoxyribonucleotide triphosphate (dNTP). Primers Amar eF and Amar eR were used in the first reaction and Amar iF and Amar eR were used in the second reaction; 0.2 µM primers and 1 IU *Taq* were used in both reactions. The first reaction used 5 µL DNA, while the second reaction used 1 µL PCR product. Ultra-pure water

(Uniscience®, USA) was added in a sufficient amount to reach a final reaction volume of 25 µL. The buffer used was 5× Colorless Gotaq® (Promega®, USA).

The PCR products were identified on 1.5% agarose gel stained with ethidium bromide. The run time was 45 minutes at 90 V.

2.11.2 The *msp*1α gene

A semi-nested PCR was performed for all samples positive for the *msp*5 gene, according to the protocol for the *msp*1α gene (LEW et al., 2002). The primers used were:

1733F: 5′-TGTGCTTATGGCAGACATTTCC-3′ **(semi-nested PCR first and second reactions)**

3134R: 5′-TCACGGTCAAAACCTTTGCTTACC-3′ **(semi-nested PCR first reaction)**

2957R: 5′-AAACCTTGTAGCCCCAACTTATCC-3′ **(semi-nested PCR second reaction)**

The PCR conditions were: denaturation at 94°C for 4 minutes, followed by 34 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 1 minute for the first reaction and at 60°C for 1 minute for the second reaction, and extension at 72°C for 2 minutes. The final extension was at 72°C for 2 minutes.

A master mix was prepared with 1× final buffer, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. Primers 1733F and 3134R were used in the first reaction, and primers 1733F and 2957R in the second reaction; 0.2 µM primers and 1 IU *Taq* were used in each reaction; . The first reaction used 5 µL of DNA and the second reaction used 1 µL PCR product. In the both reactions, ultra-pure water (Uniscience®) was added in a sufficient quantity to reach a final reaction volume of 25 µL. The buffer used was 5× Colorless Gotaq® (Promega®, USA).

The PCR products were identified on 1.5% agarose gel stained with ethidium bromide. The run time was 60 minutes at 80 V.

2.11.3 The A. phagocytophilum *msp*2 gene

A blood sample from Animal 1 and samples from culture underwent TaqMan real-time PCR to amplify a 122-bp fragment of the *A. phagocytophilum msp*2 gene. For the reaction, the probe 939p-5'-TTAAGGACAACATGCTTGTAGCTATGGAAGGCA-3' was marked at the 5′ end with the reporter dye FAM and at the 3′ end with the quencher TAMRA.

The primers used to execute the reaction were:

903f: 5′-AGTTTGACTGGAACACACCTGATC-3′

1024r: 5′-CTCGTAACCAATCTCAAGCTCAAC-3′

The PCR conditions were: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Samples with CT ("cycle threshold") of ≤40 were considered positive.

Reactions were carried out at a final volume of 12 µL composed of 1× TaqMan® Universal PCR Master Mix, 2 pmol primers 903f and 1024r, 100 pmol probe 939p (DRAZENOVICH et al., 2006), 3 µL DNA sample, and ultra-pure water in a sufficient quantity to make up the final volume.

2.11.4 The *A. bovis* 16S rRNA gene

A blood sample from Animal 1 and samples from cultures underwent PCR to amplify a 551-bp fragment of the 16S rRNA gene from *A. bovis* (KAWAHARA et al., 2006). The reaction was carried out in 1× buffer, 1.5 mM $MgCl_2$, and 0.2 mM dNTP. Here, we used 1 µM AB1F and AB1R primers, 1.25 IU Taq, and 5 µL of DNA. Ultra-pure water (Uniscience®) was added in a sufficient quantity to reach a final reaction volume of 25 µL. The buffer used in this reaction was 5× Colorless Gotaq® (Promega®, USA).

The primers used were:

AB1f: 5′-CTCGTAGCTTGCTATGAGAAC-3′

AB1r: 5′-TCTCCCGGACTCCAGTCTG-3′

The PCR conditions were: initial denaturation at 94°C for 5 minutes, followed by 39 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 1 minute and 30 seconds. The final extension was at 72°C for 7 minutes.

The PCR products were identified on 1.5% agarose gel stained with ethidium bromide. The run time was 45 minutes at 75 V.

2.11.5 The 16S rRNA gene (Anaplasmataceae)

Nested PCR was performed to amplify a 928-bp fragment of the 16S rRNA gene that can detect all organisms in the Anaplasmataceae family (BARLOUGH et al., 1996).

The primers used were:

EE-1: 5′-TCCTGGCTCAGAACGAACGCTGGCGGC-3′ } **nested PCR first reaction**

EE-2: 5′-AGTCACTGACCCAACCTTAAATGGCTG-3′

EE-3: 5′-GTCGAACGGATTATTCTTTATAGCTTGC-3′ } **nested PCR second reaction**

EE-4: 5′-CCCTTCCGTTAAGAAGGATCTAATCTCC-3′

In both reactions, the PCR conditions were: initial denaturation at 94°C for 5 minutes, followed by 39 cycles of denaturation at 94°C for 1 minute, annealing at

50°C for 2 minutes, and extension at 72°C for 1 minute and 30 seconds. The final extension was at 72°C for 7 minutes.

A master mix was prepared with 1× final buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.2 µM primers EE-1 and EE-2 (first reaction) and EE-3 and EE-4 (second reaction). It was used 1.25 IU *Taq* in both reactions; 2 µL DNA in the first reaction and 1 µL PCR product in the second reaction. Ultra-pure water (Uniscience®) was added in a sufficient quantity to reach the final reaction volume of 25 µL. The buffer used was 5× Colorless Gotaq® (Promega®, USA).

The PCR products were identified on 1.5% agarose gel stained with ethidium bromide. The run time was 45 minutes at 75 V.

2.12 Sequencing and sequence analysis

The samples positive for the *msp*1α gene and the 16S rRNA gene (Anaplasmataceae) were purified using an ExoSAP-IT purification kit (Thermo Fisher Scientific®). The purified PCR products were sequenced in both the 3′–5′ and 5′–3′ directions. The sequencing was performed at CREBIO (UNESP-Jaboticabal) in a first-generation automated sequencer (Sanger method), the ABI 3730xl DNA Analyzer (Applied Biosystems, USA).

Blood samples from Animal 1 were sequenced for the *msp*1α gene on day 1–37 after inoculation. Thereafter, positive blood samples from day 52, 196, and 258 after inoculation were selected, and their PCR products were sequenced.

Blood samples from Animal 1 were sequenced for the 16s rRNA gene on days 140, 143 and 170 after inoculation. All cultures were sequenced for the 16s rRNA gene.

Blood samples from Animal 2 were sequenced for the *msp*1α gene from day 105 (first day of positivity for the *msp*5 and *msp*1α genes) and 230.

All *msp*1α gene–positive tick organ samples from both animals were sequenced.

Two *msp*1α gene–positive culture samples were sequenced.

The *msp*1α sequences obtained were aligned using DNA Baser sequence assembly software (BioSoft®, Romania) and translated to amino acids using the ExPASy translation tool (http://web.expasy.org/translate).

For MSP1a protein tandem repeats analysis, the 5′UTR microsatellite was identified, which is located between the SD (GTAGG) sequence and the translation initiation codon (ATG) sequence (ESTRADA-PEÑA et al., 2009). The 5′UTR microsatellite structure is composed of the SD sequence (GTAGG), m (GTTT), n (GT), T, and ATG. The distance between the SD sequence and the ATG codon was calculated using the formula (4xm) + (2xn) + 1. The tandem repeats were classified according to the nomenclature proposed by DE LA FUENTE et al. (2007b).

All positive culture and blood samples for the 16S rRNA gene (Anaplasmataceae) from Animal 1 were sequenced.

3 RESULTS

3.1 Clinical and hematological evaluation of the experimental animals

Both animals did not show clinical signs after exposure to *A. marginale* strains from cell cultures. Animal 1 became positive for the *msp*5 gene on day 12 after infection and Animal 2 did not show positivity for the *msp*5 gene in PCR even 25 days after inoculation with the AmRio2 strain (first inoculation attempt). A second inoculation was carried out 28 days after the first attempt. Even so, the animal only showed a positive result on day 105 after the first infection (77 days after the second attempt). In this period, the animals were already free to graze, and were sampled monthly during this evaluation phase. Both animals did not present inclusions in the blood smears or low parasitemia. This result indicates the existence of a persistent infection.

Positivity for *msp1*α was obtained on day 14 after infection in Animal 1.

Only 59 days (day 105 after the first inoculation) after being released to pasture, Animal 2 was positive for the *A. marginale msp*5 and *msp*1α genes. This animal did not show any clinical signs throughout the study.

Both animals remained clinically stable with positivity for the *msp*5 gene via PCR throughout the study.

The parameters temperature, hematocrit, and TPP were stable throughout the experiment (Tables 1 and 2), with small deviations in situations that did not involve anaplasmosis, as in the case of the occurrence of myiasis. Inclusions in granulocytes were observed when the animals were free in the pasture (from day 46 after infection) (Figure 1). Isolation was attempted at 140 days, another two at 143 days, and one at 170 days after the inoculation.

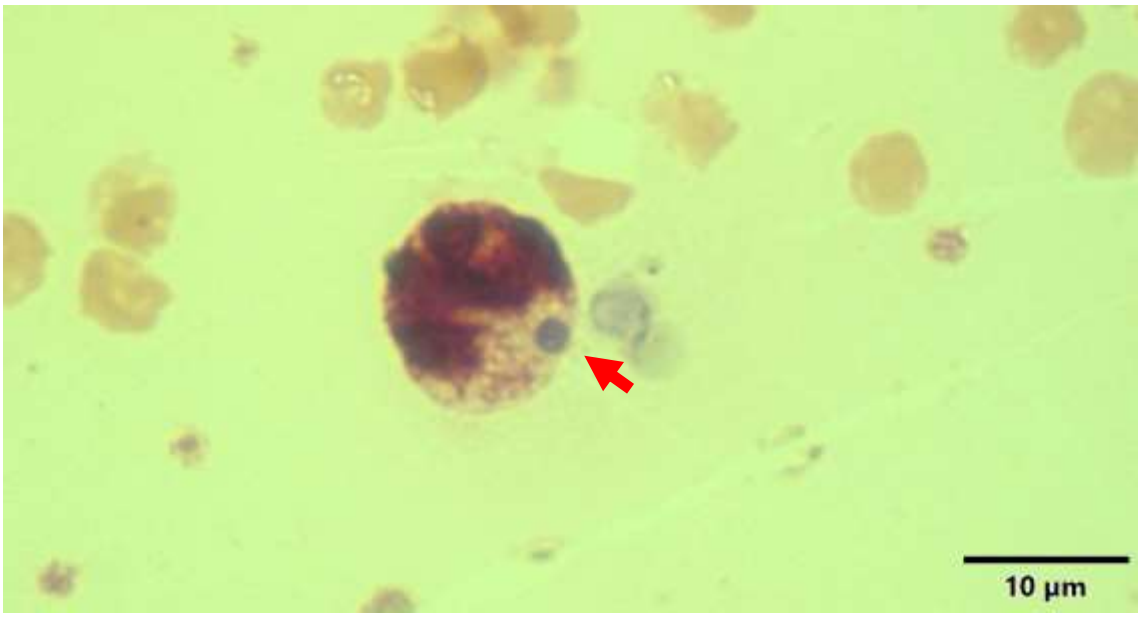

Figure 1: Basophilic inclusion with characteristics of bacterial morula inside a granulocyte (arrow) observed in a blood smear of the Animal 1. Scale bar: 10 µm.

3.1.1 Sequencing of the *msp*1α gene in blood samples

Positive samples from Animal 1 (from day 14) were sequenced continuously until 37 days after inoculation. Thereafter, PCR products were selected from positive blood samples on day 52, 196, and 258 after inoculation to obtain the sequences. The sequenced samples contained the same tandem repeats observed in the sample taken from the culture (strain AmRio1) used for the inoculum, and remained preserved (Table 4).

Blood samples from Animal 2 were sequenced on day 105 (first day of positivity for the *msp*5 and *msp*1α genes) and 230 after infection. Both led to AmRio2 as the strain found (Table 5).

Microsatellite analysis of the blood samples showed an SD–ATG distance of 23 nucleotides in all samples tested, the same distance described for both strains inoculated in the experimental infection.

3.2 Isolation of *A. marginale*

Anaplasma marginale cultures showed the first colonies containing cross-linked forms approximately 30 days after inoculation (Figure 2). The first passage

was carried out on average 50 days after infection of the IDE8 cells. After the first passage, the cells quickly reached a high level of infection and required passage at a 1:6 ratio after an average of 15 days.

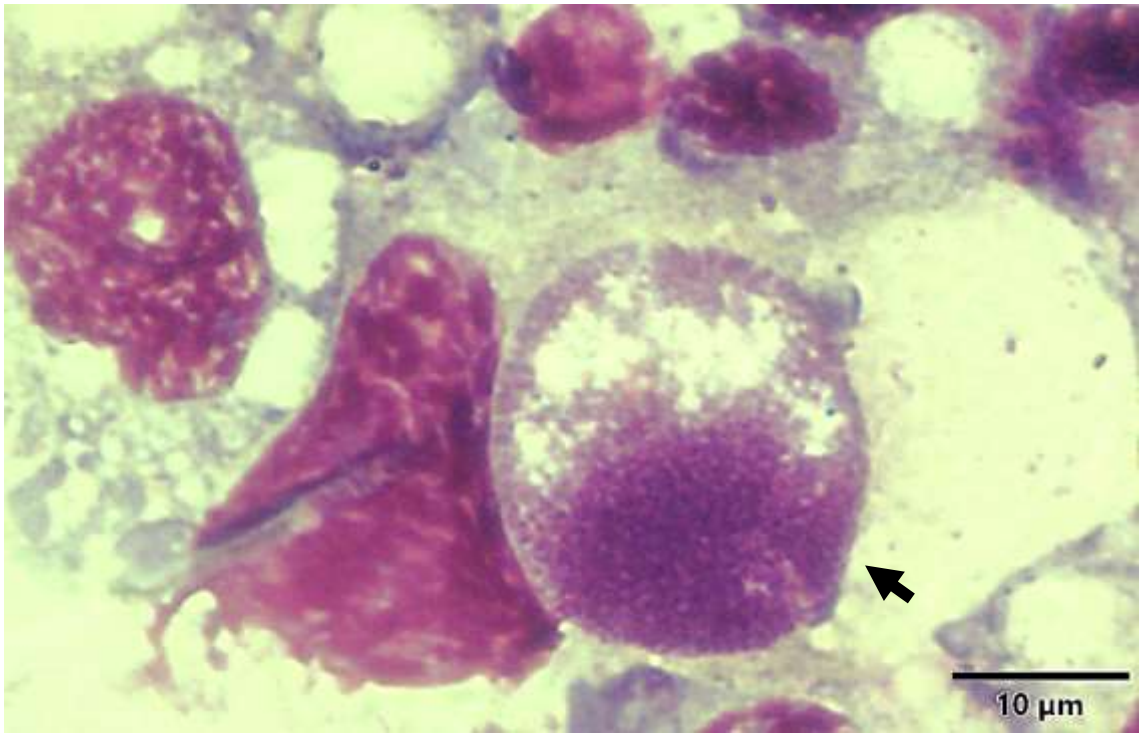

Figure 2: Giemsa-stained reticulated-forms of *A. marginale* (arrow) 30 days after culture inoculation of IDE8 cell cultures using leukocytes from Animal 1. Bar: 10 µm.

3.3 Molecular analysis of blood from Animal 1 (inoculum donor) and its cultures

The four cultures and blood samples from Animal 1 were all negative for *A. phagocytophilum* and *A. bovis*.

The culture samples were all positive for the 16S rRNA gene (Anaplasmataceae). The sequences of this gene all corresponded to the *Anaplasma marginale* species.

Subsequently, samples from the four cultures were tested and were positive for the *A. marginale msp*5 and *msp*1α genes. The *msp*1α gene from two of these samples (140 and 143 days after inoculation) were sequenced and their sequences corresponded to those of the *A. marginale* strain AmRio1 (strain inoculated in Animal 1).

The microsatellite analysis showed an SD–ATG distance of 23 nucleotides in the tested samples, the same distance described for the AmRio1 strain.

3.4 Analysis of tick samples

3.4.1 PCR

During the stage in which the animals were stabled, 32 ticks (32%) collected from Animal 1 were positive for the *msp*5 gene. Among the 32 positive ticks, six were positive in both organs (guts and salivary glands), and 26 ticks were positive in only one organ, resulting in 38 positive samples (30 gut samples and 8 salivary glands samples). From these samples, 30 were positive for the *msp*1α gene (27 gut samples and three salivary gland samples). During the confinement period, six organ samples from six different ticks (6%) (5 gut samples and 1 salivary gland sample) collected from Animal 2 were positive for the *msp*5 gene. Of these ticks, three were collected 11 days after infection (first infection) and three were collected 32 days after the first infection and 12 days after the second infection. From their organs, none was positive for the *msp*1α gene.

During the grazing period, 61 ticks (61 guts and salivary glands = 122 samples) were collected from each animal to detect the *A. marginale msp*5 gene (Table 2). From Animal 1, 26 females (42.6%) were positive for the *msp*5 gene. Among these positive ticks, five had both organs positive, i.e., guts and salivary glands, resulting in 31 positive organ samples for the *msp*5 gene. From these 31 positive samples, nine were also positive for the *msp*1α gene (five guts and four salivary gland samples). In Animal 2, 26 ticks (42.6%) were positive for the *msp*5 gene. Among these ticks, six were positive in both organs and 20 ticks were positive in a unique organ, resulting in 32 positive samples. Among these positive samples for the *msp*5 gene, six were positive also for the *msp*1α gene (five gut and one salivary gland sample). Table 3 shows the results and number of positive samples during the grazing period.

All positive samples for the *msp*1α gene were sequenced.

3.4.2 Sequencing of the *msp*1α gene in tick organs

All Animal 1 tick samples obtained during the stabled and pasture periods had MSP1a tandem repeats corresponding to the strain used in the experimental infection (AmRio1 strain) (Table 4).

All six positive tick samples obtained from Animal 2 during the pasture period resulted in *msp*1α gene sequences that corresponded to those of the strain used in the experimental infection (AmRio2 strain) (Table 5).

In the samples collected from both animals, the microsatellite analysis showed an SD–ATG distance of 23 nucleotides in all samples tested, the same distance described for both strains inoculated in the experimental infection.

Table 1: Rectal temperature, hematocrit, and TPP parameters of Animal 1, inoculated with the AmRio1 strain.

Animal 1																	
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
T°C	38	38.4	38.8	38.9	38.5	38.4	39.5	39	39.3	38.7	38.7	38.4	38.2	38.8	38.5	38.6	38.4
Ht	34	30	28	31	35	35	32	35	35	32	33	30	34	30	30	31	30
TPP					8	7	7	7.4	7.2	6.8	7	7	6.6	6.6	7	6.6	6.6
Day	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**
T°C	38.4	38.5	38.8	38.8	38.6	38.9	38.7	39	39	39	38.6	38.6	38.9	39	38.7	39	39
Ht	29	31	35	28	33	35	28	27	30	26	27	24	24	24	23	24	24
TPP	6.4	6.8	6.6	6.6	7	6.4	6.4	6.6	6.6	6.8	7	6.8	6.8	6.8	6.9	6.9	7.6
Day	**37**	**38**	**39**	**40**	**41**	**42**	**43***	**76**	**105**	**140**	**168**	**196**	**230**	**258**	**293**	**328**	**355**
T°C	38.9	38.8	38.5	38.3	37.9	39.7	38.8	39.3	39.1	39.6	39.5	39.6	32.1	39.6	41.7	38.7	40.1
Ht	23	24	24	24	27	24	26	30	33	15	29	27	25	26	23	24	23
TPP	7.2	7	7.6	7.6	7.4	7.4	7.2	7	6.8	5.7	7	7	6.8	7	7.3	6.9	7.2

* Last collection of venous blood when the animal was confined in the stall. The following collection was carried out with the animals on pasture, at monthly intervals.

Table 2: Rectal temperature, hematocrit, and TPP parameters of Animal 2, inoculated with the AmRio2 strain.

	Animal 2																	
Day	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**
T°C	38	37.9	38.3	38.6	38.1	38.4	39.7	39.6	39.1	38.7	38.8	38.6	38.2	38.3	38.5	38.5	38.3	38.6
Ht	40	34	33	38	38	39	35	38	38	34	37	36	39	31	29	31	33	34
TPP					7	7	7.3	7.6	7	6.9	6.9	6.8	6.6	6.6	6.6	7	6.6	6.6
Day	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**	**31**	**32**	**33**	**34**	**35**	**36**	**37**
T°C	38.7	38.7	38.7	38.5	38	39.1	38.8	39.4	38.9	38.8	38.9	38.6	38.8	38.6	38.6	38.3	38.2	38.6
Ht	33	34	33	36	32	36	34	34	30	35	36	33	35	31	33	35	31	33
TPP	6.6	6.6	6.6	6.8	6.2	6.6	6.6	6.8	6.2	6.4	6.4	6.4	6.4	6.7	6.6	7.4	6.2	6.4
Day	**39**	**40**	**41**	**42**	**43**	**44**	**45**	**46***	**76**	**105**	**140**	**168**	**196**	**230**	**258**	**293**	**328**	**355**
T°C	38.4	38.2	38.6	38.5	38.8	38.6	38.8	38.5	39	38.7	39.2	38.8	39.5	32.2	40.2	40.3	38.5	39.4
Ht	35	34	32	34	34	35	34	35	32	34	28	32	24	26	28	33	37	31
TPP	6.6	7.2	8.2	6.6	6.6	6.4	6.6	6.4	6.6	7	7.9	7	6.8	7	7.8	8.1	7.3	7.6

* Last collection of venous blood when the animal was confined in the stall. The following collection was carried out with the animals on pasture, at monthly intervals.

Table 3: Collection day after infection, number of ticks, and positive and negative tick organ samples for nested PCR of the *A. marginale msp*5 gene in Animal 1 and 2 during the grazing period.

Animal 1				
Day	Evaluated ticks	Positive ticks	Guts (PCR +)	Salivary glands (PCR +)
			Positive	Positive
105	6	3	1	2
148	5	1	1	-
168	10	6	4	2
210	*20 [1]	8	2	7
277	5	2	2	-
308	*5 [4]	5	5	4
337	5	-	-	-
368	5	1	1	-
Total	61	26	16	15
Animal 2				
Day	Evaluated ticks	Positive ticks	Guts (PCR +)	Salivary glands (PCR +)
			Positive	Positive
105	3	-	-	-
148	8	6	6	-
168	10	2	1	1
210	*20 [2]	8	6	4
277	*5 [2]	4	4	2
308	*5[2]	5	3	4
337	5	1	1	-
368	5	-	-	-
Total	61	26	21	11

* Samples correspond to positive PCR samples in the salivary glands and guts of the same tick. The number of ticks with both positive organs is represented in the brackets.

Table 4: Amino acids from tandem repeats of the MSP1a protein from the AmRio1 strain (BAETA et al., 2015) of *A. marginale* and its classification according to DE LA FUENTE et al. (2007b).

Sequence aminoacids (AmRio1 strain)	**Classification (DE LA FUENTE et al., 2007)**
ADSSSASGQQQESGVSSQSGQASTSSQLG	162
TDSSSASGQQQESSVSSQSGQASTSSQLG	F
TDSSSASGQQQESGVSSQSGQASTSSQLG	17
TDSSSASGQQQESSVSSQSGQASTSSQLG	F
TDSSSASGQQQESSVSSQSGQASTSSQLG	F

Table 5: Amino acids from the tandem repeats of the MSP1a protein from the AmRio2 strain (BAETA et al., 2015) of *A. marginale* and its classification according to DE LA FUENTE et al. (2007b).

Sequence aminoacids (AmRio2 strain)	**Classification (DE LA FUENTE et al., 2007)**
ADSSSASGVLSQSGQASTSSQLG	α
TDSSSAGDQQQGSGVSSQSGQASTSSQLG	β
TDSSSAGDQQQGSGVSSQSGQASTSSQLG	β
TDSSSAGDQQQGSGVSSQSGQASTSSQLG	β
TDSSSASGQQQESSVSSQSGQASTSSQLG	F

4 DISCUSSION

Both inoculated animals did not show clinical signs throughout the study, and there was no change in the parameters that characterize the acute phase of infection. This is probably because both animals had previous contact with *A. marginale* and needed antibiotic therapy before the experimental infection. Given that both animals were immunocompetent, this was probably the factor that influenced the appearance of only persistent infection characteristics in the animals. It is believed that once the ruminant is exposed to a genotype of *A. marginale*, this genotype will be the predominant one, with no success of infection on a second exposure to another strain (KOCAN et al., 2004b). This event is called the infection exclusion phenomenon (KOCAN et al., 2004b). However, a superinfection is possible when a bovine is exposed to two antigenically distinct strains (AUBRY et al., 2011). In the present study, both strains probably had antigenic characteristics similar to those previously acquired naturally by the experimental animals. Thus, there was no acute infection. In the case of the AmRio1 strain, it is known that it may cause an acute infection (SANTOS et al., 2019), so the absence of symptoms in Animal 1 was due to an immune protective factor generated by previous exposure to *A. marginale*. However, in the case of the AmRio2 strain, it is possible that the low pathogenicity of the strain may have led to the absence of clinical symptoms in Animal 2. Although the animals did not show the acute clinical pattern of anaplasmosis after experimental infection, both strains could infect them after the treatment.

It has been suggested that one of the means by which new genotypes emerge is from the sequential cycles that characterize persistent infection (PALMER et al., 2001). The analysis of MSP1a tandem repeats in the animals' blood showed no variation. (PALMER et al., 2001) observed the same result when evaluating chronically infected cattle individually. The emergence of genotypes is considered an infrequent event (PALMER et al., 2001). It is probably for this reason there is complexity in the recognition.

Animal 2 was positive for the *msp*5 gene 77 days after the second infection. Even so, six ticks from Animal 2 were positive for the *A. marginale msp*5 gene via PCR when the animal was in captivity. Animal 2 itself was negative for the same gene during this phase. One explanation for this finding is the possibility that ticks can become positive even at extremely low parasitemia. After the development of *A.*

marginale in tick gut cells, many other tissues become infected, including the salivary glands, from where the pathogen is transmitted to vertebrate hosts during the blood meal (KOCAN, 1986; KOCAN et al., 1992; GE et al., 1996). There is 95–100% probability of a tick being infected in the acute phase, while this possibility is 27–84% in the chronic phase (ERIKS et al., 1993). Replication in tick organs leads to similar concentrations of *A. marginale* in the salivary glands, regardless of the degree of rickettsemia during tick feeding (ERIKS et al., 1993).

According to (KOCAN, 1986; KOCAN et al., 1993), *A. marginale* diversity could occur during replication in the midgut and salivary glands of vector ticks. Palmer et al. (2001) found only one *msp*1α genotype when analyzing organs from 250 ticks of the *Dermacentor andersoni* species. The same authors state that this shows the frequency of occurrence of genotype emergence.

ESTRADA-PENA et al. (2009) carried out a study associating ecoregions to the circulating genotypes in the regions. They found sequences R1 (first repeat of tandem repeats) A, B, D, and alpha, as well as microsatellites C-D, G, and H in ecoregions where *R. microplus* was common and in regions where these ticks were eradicated, presenting a prevalence of transmission by *Dermacentor* spp. ticks. These results demonstrated lower selection pressure in regions where *Dermacentor* spp. were biological vectors or where they became the main vectors after the eradication of *R. microplus*. Therefore, R1 repeats would evolve under high selection pressure in places where *R. microplus* is the biological vector. At the same time, the presence of this vector would be subject to climatic factors (Estrada-Peña et al., 2009). In this study, genotype diversity was evaluated in one of the ecoregions considered to have high selective pressure, where *R. microplus* is the main vector. Even so, no variations were found in MSP1a when assessing animals and ticks individually after experimental infection.

In their results, (PALMER et al., 2001) confirm that genotypes found in one of the herds in their study had genotypes derived from a common precursor. The diversity of the MSP1a protein between strains and their tandem repeats has been described worldwide (PALMER et al., 2001; DE LA FUENTE et al., 2007b; POHL et al., 2013; MUTSHEMBELE et al., 2014; SILVA et al., 2015). Hypotheses such as various transmission events and animal movement are used to explain the variations in the amino acid length and composition in the sequences previously identified (PALMER et al., 2001; DE LA FUENTE et al., 2007b). MSP1a diversity

has been described in herds, but the emergence of new genotypes from the vertebrate host or the vector individually not been observed (PALMER et al., 2001; SANTOS et al., 2019). SANTOS et al. (2019) analyzed the occurrence of MSP1a diversity in an animal experimentally infected by the AmRio1 strain. The animal presented acute infection, and *R. microplus* ticks that were fixed during this stage of the infection were also evaluated. Both the animal and the ticks did not show MSP1a protein variation (SANTOS et al., 2019). In this study, the emergence of new genotypes in persistently infected animals and in *R. microplus* ticks was also not observed. This result confirms the statement of PALMER et al. (2001) on the emergence of new genotypes being a rare event.

The results in this study, supplemented by those of SANTOS et al. (2019), summarize the scenario regarding MSP1a sequence diversity during acute and persistent infections in individually evaluated animals and also in *R. microplus* ticks.

The re-isolation of *A. marginale* from the buffy coat generates two hypotheses: 1) After density gradient centrifugation, red blood cells contaminate cells from the interphase; 2) *A. marginale* can be found in and isolated from bovine cells other than erythrocytes. Although the first hypothesis could be considered, most samples used to isolate *A. marginale* are taken during the rickettsemia peak (MUNDERLOH et al., 1996; BLOUIN et al., 2000). It is recommended to visualize the pathogen in a blood smear in addition to PCR positivity prior to attempts at isolating it. In Animal 1, the bovine blood donor used in the present study, only a few red blood cells in whole and peripheral blood were positive, indicating persistent infection. Considering the low amount of contaminating red blood cells in the cell preparation obtained by density centrifugation of the buffy coat, the expected number of infected erythrocytes would be close to zero.

MUNDERLOH et al. (2004) propagated *A. marginale* in endothelial cells, and suggested that other cells could play a role in the biological cycle of the agent. Erythrocytes are currently the only recognized site for replication of the agent (KOCAN et al., 2010), but they cannot process antigens using the lysosomal pathways needed to process antigenic peptides for major histocompatibility complex (MHC) class II molecules (MUNDERLOH et al., 2004). They are also unable to process antigens via MHC class I molecules, which requires functional transcriptional machinery that does not exist in red blood cells (MUNDERLOH et al., 2004). The pathogenesis of bovine anaplasmosis must involve antigen-presenting

cells capable of displaying MHC class II molecules (MUNDERLOH et al., 2004). It was therefore considered that antigen-presenting cells, which are responsible for the production of immunity, should be closely linked or near the tick bite site (MUNDERLOH et al., 2004). At the time of attachment, the tick releases saliva. This induces an inflammatory response that attracts neutrophils (TATCHELL et al., 1970). Therefore, this finding suggests a second hypothesis: that, adding the results of smear observation and re-isolation, it might be possible for other cells to participate in the *A. marginale* life cycle and immune response.

5 CONCLUSIONS

The results described in this study contribute to fill a gap regarding the MSP1a diversity of *Anaplasma marginale*, considering individual characteristics of the ecoregion and the *R. microplus* vector tick.

The use of the buffy coat to isolate *A. marginale* and its characteristics related to immunogenicity when infecting cattle generates the possibility of considering the participation of another cell type in the cycle of this agent.

CHAPTER II

ISOLATION AND PROPAGATION OF A NOVEL GENOTYPE OF *Anaplasma* sp. IN IDE8 CELLS

ABSTRACT

SANTOS, Priscilla Nunes dos. **Isolation, propagation and characterization of a novel genotype of *Anaplasma* sp. in IDE8 cells.** 2020. 20p. Thesis (PhD in Veterinary Sciences). Veterinary Institute, Department of Epidemiology and Public Health, Federal Rural University of Rio de Janeiro, Seropédica, RJ, 2020.

The propagation of pathogens enables to generate information about genomics, proteomics, genetic manipulation, parasite biology and pathogen-vector interactions. The aim of this study was the propagation, identification and molecular characterization of *Anaplasma* sp., derived from bovines of Portugal, isolated in IDE8 cells. Eleven blood samples were collected from clinically healthy cattle from a farm in the municipality of Elvas, Alentejo, Portugal. Four milliliters of venous blood were collected from each animal. Blood smears were made and DNA was extracted from an aliquot of blood from each animal. One sample was selected to infect the IDE8 cells according to its result for blood smears and semi-nested PCR for the *msp*5 gene. The 16S rRNA gene was amplified in the both yielded cultures and one sample was sequenced in both directions. Subsequently, the sequence of 16S rRNA gene was aligned to related sequences to build the phylogenetic clusters. The culture was cryopreserved. Thirty-five days after the exposure of bovine blood to IDE8 cells, inclusions were observed inside the tick cells from both flasks. The inclusions morphologically resembled organisms of the Anaplasmataceae family. The rickettsia was resuscitated successfully after the cryopreservation. It was observed that the isolated microorganism revealed be accurately located in the genus Anaplasma, showing greater identity with organisms classified as *Anaplasma* sp. situated in the same cluster as *Anaplasma platys* species.

RESUMO

SANTOS, Priscilla Nunes dos. **Isolamento, propagação e caracterização de um novo genótipo de *Anaplasma* sp. em células IDE8.** 2020. 20p. Tese (Doutorado em Ciências Veterinárias). Instituto de Veterinária, Departamento de Epidemiologia e Saúde Pública, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ, 2020.

A propagação de patógenos possibilita produzir informações sobre genômica, proteômica, manipulação genética, biologia de parasitos e interações patógeno-vetor. O objetivo deste estudo foi propagar, identificar e caracterizar molecularmente *Anaplasma* sp., oriundo de bovinos de Portugal, isolados em células IDE8. Onze amostras de sangue foram coletadas de bovinos clinicamente saudáveis de uma fazenda no município de Elvas, Alentejo, Portugal. Foram coletados quatro mililitros de sangue venoso de cada animal. Esfregaços de sangue foram confeccionados e o DNA foi extraído de uma alíquota de sangue de cada animal. Uma amostra foi selecionada para a infecção de células IDE8 com base no resultado do esfregaço de sangue e da *semi-nested* PCR para o gene *msp*5. O gene 16S rRNA foi amplificado nas duas culturas preparadas e uma amostra foi enviada para sequenciamento em ambas as direções. Posteriormente, a sequência desse gene foi alinhada a sequências relacionadas para a formação dos agrupamentos filogenéticos. A cultura foi criopreservada. Observou-se inclusões nas células do carrapato de ambos os frascos trinta e cinco dias após a exposição do sangue bovino às células IDE8. Estas inclusões se apresentaram morfologicamente semelhantes aos organismos da família Anaplasmataceae. A bactéria foi ressuscitada com sucesso após criopreservação. Verificou-se que o microrganismo isolado se apresentou precisamente localizado no gênero *Anaplasma*, demonstrando maior identidade com organismos classificados como *Anaplasma* sp. situados no mesmo clado que a espécie *Anaplasma platys*.

1 INTRODUCTION

The family Anaplasmataceae comprises the genera *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia* (DUMLER et al, 2001). All members of this family are bacteria that replicate inside vacuoles originated from the membrane of the hosted eukaryotic cell (RIKIHISA, 1991). Except by the genus *Wolbachia*, all species can replicate in vertebrate hosts, and this usually occurs within mature and immature hematopoietic cells (RIKIHISA, 1991; BARBET, 1995; LOGAN et al., 1987). These cells comprise erythrocytes, reticulated cells, bone marrow-derived phagocytic cells, endothelial cells, insect cells, reproductive tissues of helminths and arthropods (DUMLER et al., 2001). At least one invertebrate vector has already been described, with a predominance of ticks playing this role for the most species studied (RIKIHISA, 1991).

The genus *Anaplasma* is composed of gram-negative, small, pleomorphic bacteria, coccoid and ellipsoidal organisms that are found inside cytoplasmic vacuoles, individually, or more often in compact inclusions (morulae) present in hematopoietic cells, mainly in myeloid cells and neutrophils, erythrocytes in peripheral blood or tissues, usually in mononuclear phagocytic cells (spleen, liver, bone marrow) from mammalian hosts. The ultrastructure of these organisms shows two morphological structures, including larger reticulated forms and smaller forms with condensed protoplasm called dense-core forms (POPOV et al., 1998). The main species of the genus *Anaplasma*, are *A. marginale*, *A. centrale*, *A. bovis*, *A. ovis*, *A. platys* and *A. phagocytophilum* (DUMLER et al., 2001).

New species of the Anaplasmataceae family have been described and including important organisms for public health and veterinary medicine (TATE et al., 2013; AGUIAR et al., 2014; ZOBBA et al., 2014; BASTOS et al., 2015; LI et al., 2015b; CABEZAS-CRUZ et al., 2016; GARCÍA-PÉREZ et al., 2016; AIT LBACHA et al., 2017; PENG et al., 2018). Among these species, many are included in the genus *Anaplasma* (TATE et al., 2013; ZOBBA et al., 2014; BASTOS et al., 2015; LI et al., 2015a; AIT LBACHA et al., 2017; PENG et al., 2018). A bacterium called *A. capra* was detected by LI et al. (2015a) and PENG et al. (2018) in samples of goats, sheeps, *Ixodes persulcatus* ticks and human patients in China. Another species of the same family was detected in Brazil and isolated by ZWEYGARTH et al. (2013) from the hemolymph of *R. microplus* tick. Subsequently, this species was called *Ehrlichia minasensis*

(CABEZAS-CRUZ et al., 2016) and revealed that this rickettsia could cause clinical signs in cattle (AGUIAR et al., 2014). Another organism belonging to Anaplasmataceae family, called *A. platys*-like, was identified by (ZOBBA et al., 2014) in ruminants from the Mediterranean region. Other organisms close to *A. platys* have been found in camels and white-tailed deer (TATE et al., 2013; BASTOS et al., 2015; AIT LBACHA et al., 2017). The description of these microorganisms contributes to the knowledge about the species that make up the Anaplasmataceae family and to the understanding of the evolutionary relationship among them.

In vitro culture systems, especially continuous cell lines derived from vectors and host tissues, play an invaluable and irreplaceable role in many aspects of research on the biology of ticks, tick-borne pathogens and disease control (BELL-SAKYI et al., 2007) The propagation pathogens *in vitro* make it possible to generate information about genetics, proteins, genetic manipulations, parasite biology and pathogen-vector interactions (BELL-SAKYI et al., 2018). Many members of the genera *Erlichia* and *Anaplasma* have been propagated in tick cell lines since 1995 (EWING, 1995; MUNDERLOH et al., 1996; BLOUIN et al., 2000; BELL-SAKYI, 2004; DUMLER et al., 2005; ZWEYGARTH et al., 2006a; TATE et al., 2013; ZWEYGARTH et al., 2013; BAETA et al., 2015), using mainly *Ixodes scapularis* cells for these studies (MUNDERLOH et al., 1994). Therefore, this chapter aims at the propagation and characterization of *Anaplasma* sp. isolated in IDE8 cells from bovines in Portugal, and its identity based on analysis of the 16S rRNA gene.

2 MATERIAL AND METHODS

2.1 Test sites and sampled animals

The experiments were conducted during a sandwich PhD (PDSE-CAPES) at the Parasitology Laboratory of the Agricultural and Veterinary Research Institute (INIAV) in Portugal, and at the Parasitic Diseases Laboratory, of the Federal Rural University of Rio de Janeiro (UFRRJ).

2.2 Blood sampling

Blood samples were collected from 11 clinically healthy cattle from a property in Elvas, in the Alentejo region, in Portugal at 18 km from Badajoz, Spain (Figure 1).

Figure 1: Image showing the place of collection and the animals collected. The cattle were crossbred and were on a property in the region of Alentejo, municipality of Elvas.

The animals were between 5 months and 2 years old. All blood samples were collected using vacuum tubes, with holder needle adapters for collection in the vacuum system. The cattle were crossbred and raised for meat production. Approximately 4 mL of jugular blood were collected from each animal in tubes with EDTA anticoagulant (BD®) and transported in an isothermal box containing recyclable ice packs to the laboratory in INIAV, Oeiras.

The blood samples were kept at 4° C until further processing. In a laminar flow chamber, an aliquot of 300 microliters of blood was transferred into polypropylene tubes for the preparation of blood smears and for the DNA extraction.

2.3 Blood smears

Air-dried blood smears were fixed in methanol for 3 minutes and stained using "Diff-Quick staining", immersing the microscope slide for 5 seconds in each solution and at the end in a solution with pH 7.2. The blood smears were examined with an optical microscope (Olympus BX40®, Japan) at a 1000-fold magnification.

2.4 DNA extraction from the blood and cultures

DNA was extracted from the blood of the five animals that showed inclusions in cells when the blood smears were examined. After *Anaplasma* sp. isolation, the DNA extraction was performed from cultures on days 21 and 35 after infection in both flasks (previously to the first subculture). Blood and culture samples were subjected to DNA extraction using the commercial kit Citogene DNA Blood Kit (Citogene®, Portugal). The sample volume used in the extraction was reduced compared to the volumes recommended by the manufacturer. For 200 µL of blood, 600 µL of "RBC Lysis Solution", 200 µL of "Cell Lysis Solution", 1 µL of RNAse A, 66 µL of "protein precipitation solution", 300 µL of isopropanol were used, 200 µL of 70% ethanol and 66 µL of "DNA hydration solution". Except for the volumes used, the extraction protocol was followed according to the manufacturer.

The samples for DNA extraction from the culture samples were taken in the flask 1 on days 106 and 119 and in the flask 2 on days 91, 96 and 110 after inoculation. The DNA extraction was caried out using the commercial kit (Promega®, USA) according to the manufacturer's recommendations for blood extraction. At this time all the cultures had undergone at least one subculture.

After extracting the DNA from the samples, they were kept at -20° C until the PCR be performed.

2.5 Polymerase Chain Reaction (PCR)

2.5.1 *msp*5 Gene

The *msp*5 gene detection was performed in DNA samples from the blood of five sampled animals who showed positivity in blood smears, and from the cultures after isolation on days 21, 35, 91, 96, 106, 110 and 119 using a semi-nested PCR as a screening for *A. marginale* detection using the following primers (ECHAIDE et al., 1998; SINGH et al., 2012):

Amar msp5 eF: 5′-GCATAGCCTCCGCGTCTTTC-3′ **(semi-nested PCR first reaction)**

Amar msp5 eR: 5′-TCCTCGCCTTGGCCCTCAGA-3′ **(semi-nested PCR first and second reactions)**

Amar msp5 iF: 5′-TACACGTGCCCTACCGAGTTA-3′ **(semi-nested PCR second reaction)**

In both reactions, the PCR conditions were: initial denaturation at 94°C for 5 minutes, followed by 34 cycles of denaturation at 94°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute. The final extension was at 72°C for 10 minutes.

A master mix was prepared with 1× final buffer, 1.5 mM $MgCl_2$, and 0.2 mM deoxyribonucleotide triphosphate (dntp). Primers Amar eF and Amar eR were used in the first reaction and Amar iF and Amar eR were used in the second reaction; 0.2 µM primers and 1 IU *Taq* were used in both reactions. The first reaction used 5 µL DNA, while the second reaction used 1 µL PCR product. Ultra-pure water (Uniscience®, USA) was added in a sufficient amount to reach a final reaction volume of 25 µL. The buffer used was the NZYTaq II with 5x Gel Load Reaction Buffer (NZYtech, Portugal®).

The PCR products were visualized on a 1.5% agarose gel plus 2 µL/100mL of GreenSafe Premium (NZYtech, Portugal®). The running time was 60 minutes at 80 V. The gel image was recorded on a photo-documenter Gel Doc EZ Imager (BIO-RAD®, USA).

2.5.2 16S rRNA Gene (Anaplasmataceae)

A *nested*-PCR was performed to amplify a 928-bp fragment of the 16S rRNA gene that can detect all organisms in the Anaplasmataceae family (BARLOUGH et al., 1996). The 16S rRNA gene was detected in DNA samples from the cultures on days 91, 96, 106, 110 and 119 after isolation.

The primers used were:

EE-1: 5′-TCCTGGCTCAGAACGAACGCTGGCGGC-3′
EE-2: 5′-AGTCACTGACCCAACCTTAAATGGCTG-3′ } **nested PCR first reaction**

EE-3: 5′-GTCGAACGGATTATTCTTTATAGCTTGC-3′
EE-4: 5′-CCCTTCCGTTAAGAAGGATCTAATCTCC-3′ } **nested PCR second reaction**

In both reactions, the PCR conditions were: initial denaturation at 94°C for 5 minutes, followed by 39 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 2 minutes, and extension at 72°C for 1 minute and 30 seconds. The final extension was at 72°C for 7 minutes.

A master mix was prepared with 1× final buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 0.2 µM primers EE-1 and EE-2 (first reaction) and EE-3 and EE-4 (second reaction). It was used 1.25 IU *Taq* in both reactions; 2 µL DNA in the first reaction and 1 µL PCR product in the second reaction. Ultra-pure water (Uniscience®) was added in a sufficient quantity to reach the final reaction volume of 25 µL. The buffer used was 5× Colorless Gotaq® (Promega®, USA).

The PCR products were identified on 1.5% agarose gel stained with ethidium bromide. The run time was 45 minutes at 75 V.

2.6 Sample selection for the IDE8 cells infection

The sample selection to infect the IDE8 cells was carried out according to its rickettsemia in blood smears (just positive blood smears were tested in PCR) and positivity in PCR.

2.7 Cell line and inoculum preparation

2.7.1 Maintenance of IDE8 cells

The cell line used was the embryonic cells of *Ixodes scapularis* (IDE8) (MUNDERLOH et al., 1994), kept in 12.5 cm² flasks, at 28 ° C in Leibovitz-15B medium supplemented with 5% Fetal Bovine Serum (Sigma-Aldrich®, USA), 10% tryptose phosphate broth, 0.1% concentrated bovine lipoprotein (MP Biomedicals®), 1% L-glutamine (200mM), 1% solution with penicillin antibiotics (10,000 IU/mL) and streptomycin (10mg/mL). The pH of the medium was adjusted to 6.6. The medium was changed weekly in a 1:2 proportion and the passage of the cells was performed when the monolayer confluence was observed. The cells were monitored using an inverted light microscope (LEICA DMIL LED, LEICA Microsystems CMS GmbH®, Germany).

2.7.2 Inoculum preparation

The selected samples (positive in the blood smear examination and in PCR) was submitted to the protocol of Blouin et al. (2000) used to isolate *A. marginale*. The blood and culture samples were processed in a laminar flow chamber. The blood was completely transferred to a sterile 15 ml conical tube (Kasvi®, Brazil) and was centrifuged at 500 x*g* for 10 minutes. The plasma and buffy coat were discarded. The erythrocytes were washed twice by centrifugations at 500 x*g* for 10 minutes and resuspended in sterile PBS (pH = 7.4). After the last wash, the supernatant was discarded and the erythrocyte pellet was distributed in 2 cryotubes, placing a volume of 0.5 ml in one (flask 1) and 1 ml in another one (flask 2). The pellets were resuspended in a 1:1 ratio in a mixture of L15B and cryoprotectant solution (DMSO 10%). The cryotubes were then placed in a Mr. Frosty container (Sigma-Aldrich®, USA) at -80 ° C.

Twenty-four hours after freezing, the cells were quickly thawed at 35° C in water bath. After thawing, the contents of each cryotube were transferred to a 15 mL conical tube. The cells were resuspended in L15B medium (PH= 6,6) and were centrifuged at 4500 x*g* for 20 minutes. The supernatant was discarded and the pellets of the each conical tubes were resuspended in L15B medium with the addition of HEPES (1M) and NaHCO3 (2.5 g / L) (PH= 7,0) (MUNDERLOH et al., 1996). The contents of each tube were

transferred to a respective 12.5 cm² culture flask containing an IDE8 cell monolayer. Twenty-four hours after the exposure of the IDE8 cells to the inoculum (infected blood), the entire culture medium was replaced in both flasks.

2.8 Culture maintenance

After the culture initiation, half of the culture medium was replaced twice a week. The cultures were checked daily by an inverted microscope (Leica DMIL Led®, Germany). Cytocentrifuge smears were prepared every 15 days.

The culture smears were made in a cytocentrifuge (Hettich Universal A®, Germany), at 1500 rpm for 5 minutes. The smears were fixed in methanol, stained with "Diff-Quick staining" (as described in topic 2.4 for blood smears) and examined under an optical microscope (Olympus BX40®, Japan) at 1000-fold magnification.

Aliquots were removed from the cultures (500 µL) for DNA extraction and PCR to confirm the infection. Culture material was collected 21 and 35 days after the culture initiation. Thereafter, samples from cultures with 91 (culture flask 1), 96 (culture flask 1), 106 (culture flask 2), 110 (culture flask 1) and 119 (culture flask 2) days after inoculation were collected. These samples were used to perform the DNA extraction.

2.9 Strain passage and cryopreservation

Initially the cells infection was close to 50%. Because of the high cell detachment from the flask surface, at this time the first subculture was performed in a ratio of 1:2 and 1:6 to 12.5cm² flasks containing a monolayer of uninfected IDE8 cells. Later, when the culture showed a stable growing, presenting more than 70% of the IDE8 cells infected, the agent was subcultured in a ratio of 1:6.

For cryopreservation, infected cells were suspended in a medium and transferred to a conical tube and centrifuged at 700 x*g* for 10 minutes. The supernatant was removed, the cell pellet was resuspended in sucrose-phosphate-glutamate buffer (SPG) (LALLINGER et al., 2010), distributed in cryotubes and frozen at -80 °C in a Mr. Frosty

container (Sigma-Aldrich®, USA). After 24 hours, these cryotubes were stored in liquid nitrogen.

After removing the cryopreserved sample from the liquid nitrogen, the tube was quickly thawed in a 35 °C water bath and the content was transferred to a 12.5cm² culture flask containing uninfected IDE8 cells. L15B culture medium supplemented with HEPES and $NaHCO_3$ (2.5 g / L) (MUNDERLOH et al., 1996) was added to maintain the infected culture.

2.10 Sequencing and phylogenetic analysis of the 16S rRNA gene

The 16s rRNA gene was amplified in a *nested*-PCR (Anaplasmataceae) and was purified using the EXOSAP-IT purification kit (Thermo Fisher Scientific®, USA). The purified PCR product was sent for sequencing in the 3'-5 'and 5'-3' directions. The sequencing was performed at CREBIO (UNESP-Jaboticabal) in a first-generation automatic sequencer (Sanger method) ABI 3730 xl DNA Analyzer (Applied Biosystems, Foster City, California, CA).

The 16s rRNA gene sequences of *Anaplasma* sp. were aligned and edited using the DNA baser sequence assembly software (BioSoft®, Romania). This sequence will be deposited in the Genbank.

The 16S rRNA gene sequence of *Anaplasma* sp. was aligned to other 16S rRNA gene sequences of related organisms in GenBank, using the tool "Basic Local Alignment Search Tool" (BLAST) for identity assessment. The 16S gene sequences of other related organisms were selected according to similarity and size.

The analysis involved 35 nucleotide sequences. All positions containing gaps and missing data were eliminated. The isolate sequence found in this study was aligned with sequences of *A. capra* (KM314237.1, KP314237.1), *A. marginale* (JQ839012.1, AF11303.1), *A. phagocytophilum* (HM366590.1, NR044762.1), *A. odocoilei* (JX876644.1, NR118489.1), *Anaplasma* sp. (AY837738.1, AY837739.1, MF76175.1), *Ehrlichia* sp. (AF18023.1), "*Candidatus* Anaplasma camelii" (KF843825.1, KX765882.1, KF843827.1) and *A. platys* (MK814421.1, MK138362.1, KX792089.2, LC269822.1, KY114935.1, EF139459.1, KX447505.1, KT982643.1) using the ClustalW

algorithm. The sequence presented a total of 879 positions in the final dataset. Sequences of *Ehrlichia ewingii* (M73227.1, NR 044747.1), *Ehrlichia canis* (EF011111.1, U26740.1), *Ehrlichia chafensis* (NR037059.1, M73222.1) and *Rickettsia typhi* (NR036948.1) were used as "outgroups" in the sequence alignment. The MEGA 6.0 software (TAMURA et al., 2013) was used to form the phylogenetic groupings of the sequences using the Maximum likelihood method based on Kimura's two-parameter model (KIMURA, 1980). A combination of phylogenetic clusters was evaluated using the Bootstrap with 1000 replicas to test different phylogenetic reconstructions.

3 RESULTS

3.1 Positive blood smears

From a total of eleven blood smears, six did not present any suggestive structure of infection by organisms belonging to the family Anaplasmataceae in erythrocytes, platelets and leukocytes. From the blood samples presenting inclusions inside of cells, only inclusions in erythrocytes were observed, considering it as a possible infection by *A. marginale*. Only one sample had more than two inclusions in erythrocytes, presenting a rickettsemia of 0.25%. Although the rickettsemia in this sample was considered low, it was higher than in the other smears.

In the PCR for the *msp*5 gene results, five samples were tested and four blood samples showed positive results. For the isolation of *Anaplasma* sp. a positive sample in PCR with a higher rickettsemia was selected.

3.2 *Anaplasma* sp. isolation and culture propagation

Thirty-five days after culture initiation, the first reticulated forms were observed in IDE8 cytocentrifuge smears (Figure 2). The first passage was made when the IDE8 cells showed a high detachment from the surface of the flask and inclusions inside the cells were observed. At this point the cells showed 50% of infection. This occurred in flask 1 (inoculated with 0.5 ml of infected erythrocytes) 51 days after the inoculation and in flask 2 (inoculated with 1 ml of infected erythrocytes) 61 days after the inoculation. Each flask was subcultured at a ratio of 1:2 and 1:6. The subcultures in both ratios developed adequately. When the cultures were presenting a stable growing, showing 70% or more of infection, subcultures were made in a ratio of 1:6. They were made every 30 days approximately. Currently, 13 passages with stable propagation and morphology have been carried out.

3.3 PCR for the *msp*5 and 16S rRNA genes (Anaplasmataceae)

A seminested PCR for the *msp*5 gene was performed on day 21 and on day 35 after inoculation. On day 21, the flask 1 (inoculum of 0.5 mL of erythrocytes) showed a negative result and the flask 2 (inoculum of 1 mL of ertythrocytes) a positive result.

Samples taken from both culture flasks at 35 days after inoculation were positive for the *msp*5 gene. The samples taken at days 91 (culture flask 2), 96 (culture flask 2), 106 (culture flask 1), 110 (culture flask 2) and 119 (culture flask 1) were negative for the *msp*5 gene.

The samples from days 91, 96, 106, 110 and 119 amplified in the PCR for the 16S rRNA gene. The sample collected on day 96 after inoculation was sent for sequencing.

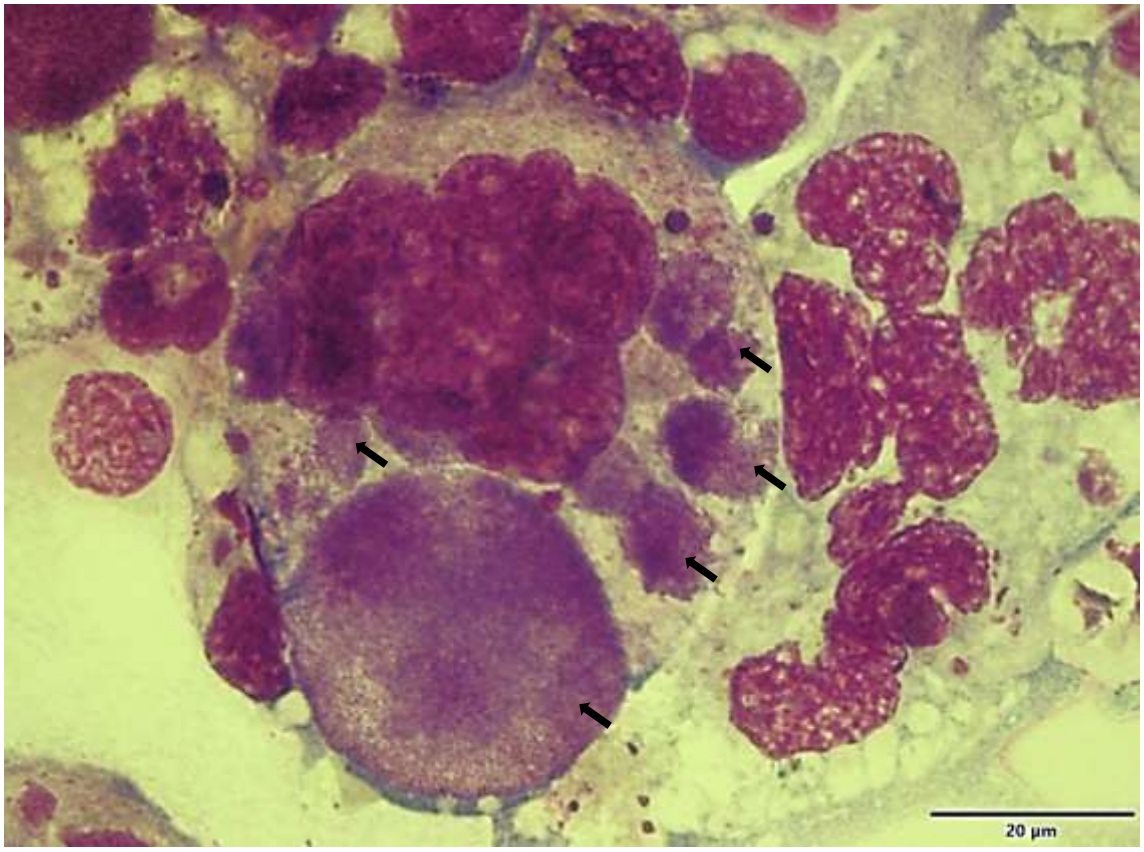

Figure 2: IDE8 cell containing multiple colonies (arrows) with reticulated forms of *A. marginale*. Staining: Panotic. Bar: 10 µm.

3.4 Cryopreservation and thawing of the isolate

Cryotubes of the stabilate were stored in liquid nitrogen using the SPG buffer. Subsequently, a cryotube was thawed to confirm the viability of the isolate. A successful

infection was macroscopically confirmed by the detachment of the monolayer cells and microscopically by inclusions in IDE8 cells. The cells reached a sufficient percentage of infection (70-80%) to carry out a subculture 22 days after thawing. The sample was cryopreserved after the passage six.

3.5 Sequencing of the 16S rRNA gene

After comparing the *Anaplasma* sp. from this study 16S rRNA gene (896bp) with other sequences deposited in the database (BLAST) high coverage and identity were observed for *Anaplasma* sp. (AY837738.1, AY837739.1) detected in the midgut of an *Anopheles arabiensis* mosquito in Kenya by Lindh et al. (2005) and *Ehrlichia* sp. (AF18023.1) detected in a Mozambique goat by BEKKER et al. (2001). Among the species well described in the literature, *Anaplasma* sp. from this study showed greater identity and coverage with *A. platys*, the causative agent of canine cyclic thrombocytopenia.

Anaplasma sp. (AY837738.1) and *Ehrlichia* sp. (AF18023.1) showed 100% identity with *Anaplasma* sp. from this study. *A. platys* species (MK814421.1, MK138362.1, KX792089.2, LC269822.1, KY114935.1, EF139459.1, KX447505.1, KT982643.1) and *Anaplasma* sp. (AY837739.1) presented 99.89% identity. While Candidatus Anaplasma camelii (KF843825.1, KX765882.1, KF843827.1), *Anaplasma* sp. (MF576175.1) and one sequence of *A. platys* (KX447505.1) showed 99.67% identity with *Anaplasma* sp. isolated in this study. All referred sequences showed 100% coverage with the *Anaplasma* sp. from this study.

In the phylogenetic tree, *Anaplasma* sp. from this study was clustered with *Anaplasma* sp. (AY837738.1, AY837739.1, MF576175.1), *Ehrlichia* sp. (AF18023.1), Candidatus Anaplasma camelii (KF843825.1, KX765882.1, KF843827.1) and *A. platys* (MK814421.1, MK138362.1, KX792089.2, LC269822.1, KY114935.1, EF139459. 1, KX447505.1, KT982643.1, KX447505.1) in the same cluster (Figure 3).

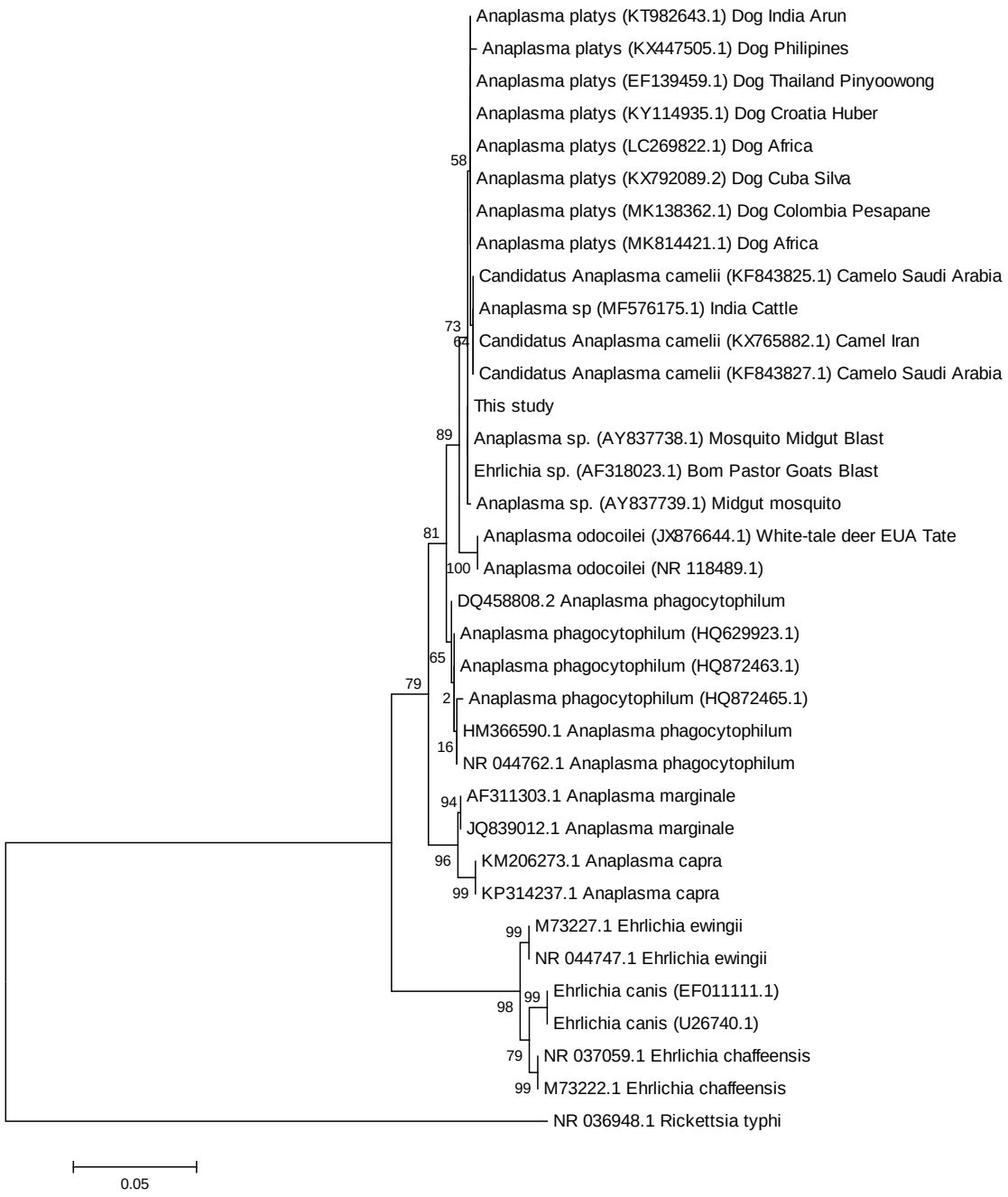

Figure 3: Molecular Phylogenetic analysis by Maximum Likelihood method. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (KIMURA, 1980). The tree with the highest log likelihood (-2316.1552) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 35 nucleotide sequences. There was a total of 879 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (TAMURA et al., 2013).

4 DISCUSSION

The development of *Anaplasma* sp. *in vitro* was similar to that observed in previous studies for other organisms of the Anaplasmataceae family (BLOUIN et al., 1998; BLOUIN et al., 2000; BASTOS et al., 2009; ZWEYGARTH et al., 2014a; BAETA et al., 2015). The infected cells showed reticulated and dense forms, similar to those that occur *in vivo* when ticks are infected (BLOUIN et al., 1998). These structures are indistinguishable between the genera *Anaplasma* and *Ehrlichia* (ZWEYGARTH et al., 2013), as well as among species belonging to the genus *Anaplasma*. In the present study, *Anaplasma* sp. is in its 13th passage, and a stability has been observed in its propagation and morphology as judged by optical microscopy.

Initially, 21 days after the culture initiation the culture flask 1 was negative and culture flask 2 showed a positive result for the *msp*5 gene in PCR. Culture flask 1 showed positivity for the *msp*5 gene on day 35, and at this time both culture flasks showed reticulated forms in cytocentrifuge smears. The difference between flasks 1 and 2 was the amount of red blood cells used to infect IDE8 cells in each flask. A possible explanation for the negative result in the first PCR in flask 1 is the presence of inhibitors remaining after the DNA extraction from the culture sample, or even the total DNA present in an amount that can interfere in the target DNA detection. This finding about PCR inhibition is observed by other authors, who confirm that PCR inhibitors are a very heterogeneous group of chemical substances (SCHRADER et al., 2012). These can be organic or inorganic substances, dissolved or in a solid state. The best know PCR inhibitors are organic substances such as bile salts, urea, phenol, ethanol, polysaccharides, sodium dodecyl sulfate (SDS), humic acid, tannic acid, melanin, as well as different proteins such as collagen, myoglobin, hemoglobin, lactoferrin, immunoglobulin G (IgG) and proteinases (ROSSEN et al., 1992; RÅDSTRÖM et al., 2004; SCHRADER et al., 2012). Exogenous DNA can also affect in the PCR effectiveness due to competition with the target DNA during the PCR execution (SCHRADER et al., 2012).

The positive result on day 35 was probably due to an growth in *A. marginale in vitro*, which made it possible to detect the DNA of this organism in the sample. At this point a co-infection probably occurred in both cultures, with the growth of *A. marginale* and *Anaplasma* sp. simultaneously. When analyzing infected tick cells by optical microscopy, all organisms in the Anaplasmataceae family have a similar morphology.

Samples of days 91, 96, 106, 110 and 119 were all negative for the *msp*5 gene of *A. marginale* and were positive for the 16S rRNA gene. Throughout the passages there was a probable predominance of *Anaplasma* sp., until the complete disappearance of *A. marginale*.

The use of an inoculum with a rickettsemia of 0.25% for *A. marginal* did not interfere with the proliferation of *Anaplasma* sp. in IDE8 cells. For the isolation of *A. marginale*, MUNDERLOH et al. (1996) and BLOUIN et al. (2000) suggest the use of blood in peak rickettsemia. Other studies which successful results in isolating the agent had inoculum ranging from 10% to 64% of rickettsemia (MUNDERLOH et al., 1996; BLOUIN et al., 2000; BASTOS et al., 2009; BAETA et al., 2015). Therefore, if the rickettsemia for *A. marginale* was higher, probably this agent would have prevailed.

Other authors described organisms with an identity close to *A. platys*, showing tropism for cells such as monocytes and neutrophils, and not just platelets (KOCAN, 1986; DE LA FUENTE et al., 2001c; LIU et al., 2012; TATE et al., 2013; ZOBBA et al., 2014). ZOBBA et al. (2014) described the occurrence of neuthrophil-tropic strains closely related to *A. platys*. These organisms did not infect platelets. The tropism of *A. odocoilei* isolated from white-tailed deer (*Odocoileus virginianus*), to platelets was verified by optical and electronic microscopy, in situ hybridization, immunohistochemistry and PCR in purified platelets (TATE et al., 2013). TATE et al. (2013) also detected DNA from *A. odocoilei* in monocytes, creating theories for this finding such as: the infection of the agent in both cell types; the presence of contaminating platelets in the purified monocyte preparation; the phagocytosis of infected platelets by monocytes or the releasing of organisms during the sample processing. In this study, it was not found inclusions in platelets and white cells, or inclusions in erythrocytes morphologically different from those known for *A. marginale*. The inoculum used for the IDE8 cells infection consisted of washed erythrocytes pellets after the removal of the buffy coat and the plasma, in addition to washing it. Given the nature of the inoculum, the hypotheses that would better explain the origin of the organism would be: I) the presence of platelets or white cells that contaminated the purified erythrocyte preparation; II) the presence of free organisms among erythrocytes. However, the last hypothesis seems unlikely due to the speed of rotations used for the inoculum preparation. Lis et al. (2014) proposed the speed of 210 x*g* for the separation of cells and cellular debris, while the speed of 20,000 x*g* was used to obtain purified *A. marginale* from IDE8 cells.

Anaplasma platys is the causative agent of canine infectious cyclic thrombocytopenia. Thrombocytopenia occurs as a consequence of the combination of direct injury to platelets and immunomediated mechanisms, tending to spontaneous recovery of the host (HARVEY et al., 1978). Symptoms in dogs are usually fever, lethargy, decreasing appetite, weight loss, pale mucous membranes, petechial hemorrhages in skin and oral mucosa, epistaxis and lymphadenomegaly, although most animals are asymptomatic when infected (BRADFIELD et al., 1996; BOUZOURAA et al., 2016). Similarly, there are few reports of animals infected with organisms that are genetically related to *A. platys* causing symptoms. TATE et al. (2013) experimentally infected deer with *A. odocoilei* and observed trombocytopenia, while other clinical parameters did not change. In the studies by BASTOS et al. (2015) and SAID et al. (2018) in Saudi Arabia and North Africa, respectively, camels positive for "Candidatus Anaplasma camelii" did not show clinical signs. AIT LBACHA et al. (2017) characterized Anaplasmataceae strains circulating in the dromedary camels showing clinical sings in Morocco. Nevertheless, it could not be affirmed that the origin of the symptoms was the infection by this rickettsia (AIT LBACHA et al., 2017). In cattle, ZOBBA et al. (2014) and DAHMANI et al. (2015) did not mention any symptoms that occurred in animals positive for *A. platys*-like organisms. In this study, all animals examined were clinically healthy.

In this study, an organism with a high identity with *A. platys* was found and isolated from bovine blood. The most known hosts of *A. platys* are dogs, but this pathogen has been found in cats, foxes (*Vulpes vulpes*), camels (*Camelus bactrianus*), red deer, sika deer, cattle and humans (HARVEY et al., 1978; MAGGI et al., 2013; QUROLLO et al., 2014; CARDOSO et al., 2015; DAHMANI et al., 2015; LI et al., 2015a; LI et al., 2015b). *Rhipicephalus sanguineus* ticks are currently considered to be the *A. platys* vector (WOODY et al., 1991) although this tick could not transfer the agent to naive dogs (SIMPSON et al., 1991).

In this study *Anaplasma* sp. from bovine blood was in the same cluster as *A. platys*. Similar findings have been reported by other authors, who described organisms showing high identity to *A. platys* in cattle in Italy, Algeria and India (ZOBBA et al., 2014; DAHMANI et al., 2015; ROY et al., 2018) and in other ruminants such as goats and sheep in Italy, goats in Mozambique and China (BEKKER et al., 2001; LIU et al., 2012;

ZOBBA et al., 2014), camels in Saudi Arabia, Morocco and North Africa (MAZZOLA et al., 1976; RÅDSTRÖM et al., 2004; BASTOS et al., 2015; BEN SAID et al., 2015; AIT LBACHA et al., 2017) and goats and deers in the USA (TATE et al., 2013). *Anaplasma platys* not only plays an important role in veterinary medicine (MURPHY et al., 1998; DAGNONE et al., 2001; DUMLER et al., 2001), but has also proven to be an important agent in public health (ARRAGA-ALVARADO et al., 1999; TAMÍ et al., 2002; MAGGI et al., 2013). This study is the first to detect *Anaplasma* sp. closely related to *A. platys* in Portugal, and also the first to isolate and propagate this organism *in vitro* in Europe.

There are also reports that this bacterium was detected in the gut of *Anopheles arabiensis* mosquitoes (LINDH et al., 2005) and in *Hyalloma truncantum* ticks (ALLSOPP et al., 1997). These results could indicate the role of these arthropods as vectors since no arthropod was identified as a vector of *A. platys* and *A. platys*-like organisms. At the same time, it cannot be determined that the detected *A. platys*-like DNA does not come from the blood of the vertebrate host, which could has remained in the digestive tract of the arthropods (ALLSOPP et al., 1997; LINDH et al., 2005).

The *A. platys*-like strains from Italy were phylogenetically evaluated using the 16S rRNA and GroEL genes (ZOBBA et al., 2014) These organisms were genetically similar to *A. platys* sharing 92 to 93% of identity in GroEL gene. They had a weaker relationship to *A. phagocytophilum*, and showed 79 to 80% of identity. Despite the weaker proximity to *A. phagocytophilum*, this rickettsia was found in ruminants and showed greater tropism for granulocytes (cells that are usually infected with *A. phagocytophilum*) (ZOBBA et al., 2014). Thus, ZOBBA et al. (2014) suggested the possibility that *A. platys*-like is an ancestor of *A. platys* with host and cell tropism more similar to those of *A. phagocytophilum*, infecting neutrophils of both carnivores and ruminants. *Anaplasma platys* could have originated from *A. phagocytophilum*-like strains by specialization in the host range (from tropism to multiple hosts to tropism exclusively to carnivores) and by a transition from cellular tropism, from granulocytes to platelets. (ZOBBA et al., 2014). Regarding this background, *A. platys*-like could represent an ancestor of strains of *A. platys* in ruminants that have retained their original tropism for cells and hosts.

Tick cell lines have become increasingly important tools in research (BELL-SAKYI et al., 2007; BELL-SAKYI et al., 2018), facilitating studies in many aspects of the biology, physiology and control of ticks and tick-borne pathogens (BELL-SAKYI et

al., 2018). These cell lines have been used extensively in the isolation, propagation and study of tick-borne bacteria and viruses, and for a large variety of studies on the host-pathogen interaction, as well as on genomic and proteomic interactions (BLOUIN et al., 2002; BELL-SAKYI et al., 2007; ALBERDI et al., 2015; BAETA et al., 2015; GULIA-NUSS et al., 2016; CABEZAS-CRUZ et al., 2017). Due to the wide range of tick species represented by tick cell lines, it is easier to determine which tick cell line will be the best to propagate bacterial species in particular, which can range from easier cultivable species, such as *Ehrlichia* spp. to species such as *A. centrale*, which have fastidious cultivation (BELL-SAKYI et al., 2018). The ability of tick cells to survive many months without subcultures facilitates the isolation of slow-growing and laborious bacteria, which may be present at very low levels in the inoculum (BELL-SAKYI et al., 2018). Thus, the isolation and propagation of *Anaplasma* sp. in IDE8 cells guarantees the production of quality material for studies on biology, pathogen-host interaction, morphology, and its taxonomic classification.

Ruminants can be the key in the epidemiology of the genus *Anaplasma*, and act as hosts of the infection or reservoirs, as all species of the genus infect domestic and wild ruminants worldwide (ZOBBA et al., 2014; DAHMANI et al., 2015). The isolation of the *Anaplasma* sp. described here, may contribute to a better understanding of the evolutionary relationships within the genus *Anaplasma* and the role of ruminants in this process.

5 CONCLUSIONS

The isolation and culture propagation of an *Anaplasma* species obtained from indigenous cattle from Portugal, has been described.

The cultures showed a morphology and a development comparable to other members of the Anaplasmataceae family. Currently the culture is in its 13th subculture.

In the phylogenetic analysis of the 16S rRNA gene, *Anaplasma* sp. is in the same cluster as organisms which are closely related to *A. platys*-like of ruminants and to the species *A. platys* of carnivores.

This study was the first in Portugal to detect and the first in Europe to isolate and propagate an *Anaplasma* sp. genetically related to *A. platys* from a ruminant.

6 REFERENCES

Abba, Y.Jesse, F.Sadiq, M.Ibrahim, H.Chung, E.Bitrus, A. et al. Clinical management and gross pathological findings of a severe anaplamosis in a dairy cow. **Journal of Advace in Veterinary and Animal Research**, 3, n. 2, p. 195-199, 2016.

Abdelkadir, K.; Palomar, A.M.; Portillo, A.; Oteo, J.A.; Ait-Oudhia, K.; Khelef, D. Presence of Rickettsia aeschlimannii, 'Candidatus Rickettsia barbariae' and Coxiella burnetii in ticks from livestock in Northwestern Algeria. **Ticks and Tick-borne Diseases**, 10, n. 4, p. 924-928, 2019/06/01/ 2019.

Aguiar, D.M.Ziliani, T.F.Zhang, X.Melo, A.L.Braga, I.A.Witter, R. et al. A novel Ehrlichia genotype strain distinguished by the TRP36 gene naturally infects cattle in Brazil and causes clinical manifestations associated with ehrlichiosis. **Ticks Tick Borne Dis.**, 5, n. 5, p. 537-544, Sep 2014.

Aguirre, D.H.; Gaido, A.B.; Vinabal, A.E.; De Echaide, S.T.; Guglielmone, A.A. Transmission of *Anaplasma marginale* with adult Boophilus microplus ticks fed as nymphs on calves with different levels of rickettsaemia. **Parasite**, 1, n. 4, p. 405-407, 12// 1994. 10.1051/parasite/1994014405.

Aguirre, E.; Tesouro, M.; Ruiz, L.; Amusategui, I.; Sainz, A. Genetic characterization of Anaplasma (Ehrlichia) platys in dogs in Spain. **Journal of Veterinary Medicine, Series B**, 53, n. 4, p. 197-200, 2006.

Ait Lbacha, H.Zouagui, Z.Alali, S.Rhalem, A.Petit, E.Ducrotoy, M.J. et al. "Candidatus *anaplasma camelii*" in one-humped camels (*Camelus dromedarius*) in Morocco: a novel and emerging *anaplasma* species? **Infectious Diseases of Poverty**, 6, n. 1, p. 1-1, 2017.

Alberdi, M.P.; Nijhof, A.M.; Jongejan, F.; Bell-Sakyi, L. Tick cell culture isolation and growth of Rickettsia raoultii from Dutch Dermacentor reticulatus ticks. **Ticks and Tick-borne Diseases**, 3, n. 5–6, p. 349-354, 12// 2012.

Alberdi, P.Ayllón, N.Cabezas-Cruz, A.Bell-Sakyi, L.Zweygarth, E.Stuen, S. et al. Infection of Ixodes spp. tick cells with different Anaplasma phagocytophilum isolates induces the inhibition of apoptotic cell death. **Ticks and tick-borne diseases**, 6, n. 6, p. 758-767, 2015.

Alleman, A.R.; Palmer, G.H.; McGuire, T.C.; McElwain, T.F.; Perryman, L.E.; Barbet, A.F. *Anaplasma marginale* major surface protein 3 is encoded by a polymorphic, multigene family. **Infection and Immunity**, 65, n. 1, p. 156-163, 1997.

Alleman, R.; Kamper, M.S.; Viseshakul, N.; Barbet, A.F. Analysis of the *Anaplasma marginale* genome by pulsed-field electrophoresis. **Microbiology**, 139, n. 10, p. 2439-2444, 1993.

Allred, D.R.McGuire, T.C.Palmer, G.H.Leib, S.R.Harkins, T.M.McElwain, T.F. et al. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. **Proc. Natl. Acad. Sci. U.S.A.**, 87, n. 8, p. 3220-3224, April 1, 1990 1990.

Allsopp, M.; Visser, E.; Du Plessis, J.; Vogel, S.; Allsopp, B. Different organisms associated with heartwater as shown by analysis of 16S ribosomal RNA gene sequences. **Veterinary Parasitology**, 71, n. 4, p. 283-300, 1997.

Anderson, J.F. The natural history of ticks. **Medical Clinics**, 86, n. 2, p. 205-218, 2002.

Antognoni, M.T.; Veronesi, F.; Morganti, G.; Mangili, V.; Fruganti, G.; Miglio, A. Natural infection of Anaplasma platys in dogs from Umbria region (Central Italy). **Vet Ital**, 50, n. 1, p. 49-56, 2014.

Araujo, F.R.; Madruga, C.R.; Soares, C.O.; Kessler, R.H. Progressos na imunização contra *Anaplasma marginale*. **Pesquisa Veterinaria Brasileira**, 23, n. 4, p. 139-148, 2003.

Arraga-Alvarado, C.; Palmar, M.; Parra, O.; Salas, P. Fine structural characterisation of a Rickettsia-like organism in human platelets from patients with symptoms of ehrlichiosis. **Journal of Medical Microbiology**, 48, n. 11, p. 991-997, 1999.

Atif, F.A. Alpha proteobacteria of genus Anaplasma (Rickettsiales: Anaplasmataceae): Epidemiology and characteristics of Anaplasma species related to veterinary and public health importance. **Parasitology**, 143, n. 6, p. 659-685, 2016.

Aubry, P.; Geale, D.W. A review of bovine anaplasmosis. **Transbound EmergingDisesase**, 58, n. 1, p. 1-30, Feb 2011.

Baeta, B.A.Ribeiro, C.C.Teixeira, R.C.Cabezas-Cruz, A.Passos, L.M.Zweygarth, E. et al. Characterization of two strains of *Anaplasma marginale* isolated from cattle in Rio de Janeiro, Brazil, after propagation in tick cell culture. **Ticks Tick Borne Dis.**, 6, n. 2, p. 141-145, Mar 2015.

Bakken, J.S.; Krueth, J.; Wilson-Nordskog, C.; Tilden, R.L.; Asanovich, K.; Dumler, J.S. Clinical and Laboratory Characteristics of Human Granulocytic Ehrlichiosis. **JAMA**, 275, n. 3, p. 199-205, 1996.

Barbet, A.; Blentlinger, R.; Yi, J.; Lundgren, A.; Blouin, E.; Kocan, K. Comparison of surface proteins of *Anaplasma marginale* grown in tick cell culture, tick salivary glands, and cattle. **Infection and Immunity**, 67, n. 1, p. 102-107, 1999.

Barbet, A.; Yi, J.; Lundgren, A.; McEwen, B.; Blouin, E.; Kocan, K. Antigenic variation of *Anaplasma marginale*: major surface protein 2 diversity during cyclic transmission between ticks and cattle. **Infection and Immunity**, 69, n. 5, p. 3057-3066, 2001.

Barbet, A.F. Recent developments in the molecular biology of anaplasmosis. **Veterinary Parasitology**, 57, n. 1–3, p. 43-49, 3// 1995.

Barbet, A.F.; Palmer, G.H.; Myler, P.J.; McGuire, T.C. Characterization of an Immunoprotective Protein Complex of *Anaplasma marginale* by Cloning and Expression of the Gene Coding for Polypeptide Am1O5L **Infection and Immunity**, 55, n. 10, p. 2428-2435, 1987.

Barlough, J.E.; Madigan, J.E.; DeRock, E.; Bigornia, L. Nested polymerase chain reaction for detection of *Ehrlichia equi* genomic DNA in horses and ticks (Ixodes pacificus). **Vet. Parasit.**, 63, n. 3, p. 319-329, 1996/06/01/ 1996.

Bastos, A.D.; Mohammed, O.B.; Bennett, N.C.; Petevinos, C.; Alagaili, A.N. Molecular detection of novel Anaplasmataceae closely related to *Anaplasma platys* and *Ehrlichia canis* in the dromedary camel (*Camelus dromedarius*). **Vet. Microb.**, 179, n. 3-4, p. 310-314, 2015.

Bastos, C.V.; Passos, L.M.F.; Vasconcelos, M.M.C.; Ribeiro, M.F.B. In Vitro establishment and propagation of a brazilian strain of *Anaplasma marginale* with appendage in IDE8 (*Ixodes scapularis*) cells. **Brazilian Journal of Microbiology**, 40, n. 2, p. 399-403, 2009.

Battilani, M.; De Arcangeli, S.; Balboni, A.; Dondi, F. Genetic diversity and molecular epidemiology of Anaplasma. **Infection, Genetics and Evolution**, 49, p. 195-211, 2017/04/01/ 2017.

Bekker, C.P.; Vink, D.; Pereira, C.M.L.; Wapenaar, W.; Langa, A.; Jongejan, F. Heartwater (Cowdria ruminantiumInfection) as a Cause of Postrestocking Mortality of Goats in Mozambique. **Clinical and Diagnostic Laboratory Immunology**, 8, n. 4, p. 843-846, 2001.

Bekker, C.P.J.Postigo, M.Taoufik, A.Bell-Sakyi, L.Ferraz, C.Martinez, D. et al. Transcription Analysis of the Major Antigenic Protein 1 Multigene Family of Three In Vitro-Cultured Ehrlichia ruminantium Isolates. **Journal of Bacteriology**, 187, n. 14, p. 4782, 2005.

Bell-Sakyi, L. Ehrlichia ruminantium grows in cell lines from four ixodid tick genera. **Journal of Comparative Pathology**, 130, n. 4, p. 285-293, May 2004.

Bell-Sakyi, L.; Darby, A.; Baylis, M.; Makepeace, B.L. The Tick Cell Biobank: A global resource for in vitro research on ticks, other arthropods and the pathogens they transmit. **Ticks and Tick Borne Diseases**, 9, n. 5, p. 1364-1371, Jul 2018.

Bell-Sakyi, L.; Zweygarth, E.; Blouin, E.F.; Gould, E.A.; Jongejan, F. Tick cell lines: tools for tick and tick-borne disease research. **Trends in Parasitology**, 23, n. 9, p. 450-457, 2007.

Ben Said, M.Belkahia, H.Karaoud, M.Bousrih, M.Yahiaoui, M.Daaloul-Jedidi, M. et al. First molecular survey of *Anaplasma bovis* in small ruminants from Tunisia. **Vet. Microb.**, 179, n. 3-4, p. 322-326, 2015.

Blouin, E.F.; Barbet, A.F.; Yi, J.; Kocan, K.M.; Saliki, J.T. Establishment and characterization of an Oklahoma isolate of *Anaplasma marginale* in cultured *Ixodes scapularis* cells. **Veterinary Parasitology**, 87, n. 4, p. 301-313, 2000.

Blouin, E.F.; de la Fuente, J.; Garcia-Garcia, J.C.; Sauer, J.R.; Saliki, J.T.; Kocan, K.M. Applications of a cell culture system for studying the interaction of *Anaplasma marginale* with tick cells. **Animal Health Research Reviews**, 3, n. 02, p. 57-68, 2002.

Blouin, E.F.; Saliki, J.T.; Kocan, K.M.; Rodgers, S.J. Evaluation of *Anaplasma marginale* from Tick Cell Culture as an Immunogen for Cattle. **Annals of the New York Academy of Sciences**, 849, n. 1, p. 253-258, 1998.

Borjesson, D.L.; Simon, S.I.; Hodzic, E.; DeCock, H.E.; Ballantyne, C.M.; Barthold, S.W. Roles of neutrophil β2 integrins in kinetics of bacteremia, extravasation, and tick acquisition of Anaplasma phagocytophila in mice. **Blood, The Journal of the American Society of Hematology**, 101, n. 8, p. 3257-3264, 2003.

Bouzouraa, T.René-Martellet, M.Chêne, J.Attipa, C.Lebert, I.Chalvet-Monfray, K. et al. Clinical and laboratory features of canine *Anaplasma platys* infection in 32 naturally infected dogs in the Mediterranean basin. **Ticks Tick-borne Dis**, 7, n. 6, p. 1256-1264, 2016.

Bowie, M.V.; de la Fuente, J.; Kocan, K.M.; Blouin, E.F.; Barbet, A.F. Conservation of major surface protein 1 genes of *Anaplasma marginale* during cyclic transmission between ticks and cattle. **Gene**, 282, n. 1–2, p. 95-102, 1/9/ 2002.

Boynton, W.H.; Woods, G.M. Deer as carriers of anaplasmosis. **Science (Washington)**, 78, n. 2033, 1933.

Bradfield, J.F.; Vore, S.J.; Pryor, W.H., Jr. Ehrlichia platys infection in dogs. **Laboratory Animal Science**, 46, n. 5, p. 565-568, Oct 1996.

Brown, W.C.McGuire, T.C.Mwangi, W.Kegerreis, K.A.Macmillan, H.Lewin, H.A. et al. Major histocompatibility complex class II DR-restricted memory CD4(+) T lymphocytes recognize conserved immunodominant epitopes of *Anaplasma marginale* major surface protein 1a. **Infection and Immunity**, 70, n. 10, p. 5521-5532, Oct 2002.

Brown, W.C.; McGuire, T.C.; Zhu, D.; Lewin, H.A.; Sosnow, J.; Palmer, G.H. Highly conserved regions of the immunodominant major surface protein 2 of the genogroup II ehrlichial pathogen *Anaplasma marginale* are rich in naturally derived CD4+ T lymphocyte epitopes that elicit strong recall responses. **Journal of Immunology**, 166, n. 2, p. 1114-1124, Jan 15 2001a.

Brown, W.C.; Palmer, G.H.; Lewin, H.A.; McGuire, T.C. CD4(+) T lymphocytes from calves immunized with *Anaplasma marginale* major surface protein 1 (MSP1), a heteromeric complex of MSP1a and MSP1b, preferentially recognize the MSP1a carboxyl terminus that is conserved among strains. **Infection and Immunity**, 69, n. 11, p. 6853-6862, Nov 2001b.

Cabezas-Cruz, A.; Alberdi, P.; Valdés, J.J.; Villar, M.; de la Fuente, J. Anaplasma phagocytophilum infection subverts carbohydrate metabolic pathways in the tick vector, Ixodes scapularis. **Frontiers in cellular and infection microbiology**, 7, p. 23, 2017.

Cabezas-Cruz, A.; de la Fuente, J. *Anaplasma marginale* major surface protein 1a: a marker of strain diversity with implications for control of bovine anaplasmosis. **Ticks and tick-borne diseases**, 6, n. 3, p. 205-210, 2015/04// 2015.

Cabezas-Cruz, A.Passos, L.M.F.Lis, K.Kenneil, R.Valdés, J.J.Ferrolho, J. et al. Functional and Immunological Relevance of *Anaplasma marginale* Major Surface Protein 1a Sequence and Structural Analysis. **PLoS ONE**, 8, n. 6, p. e65243, 2013a.

Cabezas-Cruz, A.; Vancová, M.; Zweygarth, E.; Ribeiro, M.F.B.; Grubhoffer, L.; Passos, L.M.F. Ultrastructure of *Ehrlichia mineirensis*, a new member of the *Ehrlichia* genus. **Veterinary Microbiology**, 167, n. 3-4, p. 455-458, 2013b.

Cabezas-Cruz, A.Zweygarth, E.Vancova, M.Broniszewska, M.Grubhoffer, L.Passos, L.M. et al. *Ehrlichia minasensis* sp. nov., a new species within the genus Ehrlichia isolated from the tick *Rhipicephalus microplus*. **International Journal of Systematic and Evolutionary Microbiology**, Jan 12 2016.

Camacho-Nuez, M.; de Lourdes Muñoz, M.; Suarez, C.E.; McGuire, T.C.; Brown, W.C.; Palmer, G.H. Expression of polymorphic msp1β genes during acute Anaplasma marginale rickettsemia. **Infection and Immunity**, 68, n. 4, p. 1946-1952, 2000.

Cardoso, L.Gilad, M.Cortes, H.C.E.Nachum-Biala, Y.Lopes, A.P.Vila-Viçosa, M.J. et al. First report of Anaplasma platys infection in red foxes (Vulpes vulpes) and molecular detection of Ehrlichia canis and Leishmania infantum in foxes from Portugal. **Parasites & Vectors**, 8, n. 1, p. 144, 2015/03/23 2015.

Ceci, L.; Iarussi, F.; Greco, B.; Lacinio, R.; Fornelli, S.; Carelli, G. Retrospective Study of Hemoparasites in Cattle in Southern Italy by Reverse Line Blot Hybridization. **Journal of Veterinary Medical Science**, 76, n. 6, p. 869, 2014.

Chomel, B.B.; Carniciu, M.L.; Kasten, R.W.; Castelli, P.M.; Work, T.M.; Jessup, D.A. Antibody prevalence of eight ruminant infectious diseases in California mule and black-tailed deer (*Odocoileus hemionus*). **Journal of Wildlife Diseases**, 30, n. 1, p. 51-59, Jan 1994.

Christensen, J.; Osebold, J.; Harrold, J.B.; Rosen, M.N. Persistence of latent *Anaplasma marginale* infection in deer. **Journal of the American Veterinary Medical Association**, 136, p. 426-427, 1960.

Coetzee, J.F.; Apleya, M.D.; Kocanb, K.M.; Rurangirwac, F.R.; Van Donkersgoedd, J. Comparison of three oxytetracycline regimens for the treatment of persistent *Anaplasma marginale* infections in beef cattle. **Veterinary Parasitology**, 127, n. 1, p. 61-73, 1/4/ 2005.

Dagnone, A.S.; De Morais, H.S.A.; Vidotto, M.C.; Jojima, F.S.; Vidotto, O. Ehrlichiosis in anemic, thrombocytopenic, or tick-infested dogs from a hospital population in South Brazil. **Veterinary Parasitology**, 117, n. 4, p. 285-290, 2003.

Dagnone, A.S.; de Morais, H.S.A.; Vidotto, O. Animal and human Ehrlichiosis/Erliquiose nos animais e no homem. **Semina: Ciências Agrárias**, 2001.

Dahmani, M.; Loudahi, A.; Mediannikov, O.; Fenollar, F.; Raoult, D.; Davoust, B. Molecular detection of *Anaplasma platys* and *Ehrlichia canis* in dogs from Kabylie, Algeria. **Ticks Tick-borne Dis.**, 6, n. 2, p. 198-203, 2015/03/01/ 2015.

Davidson, W.; Goff, W.; Williams, E.; Barker, I. Anaplasmosis. *In*: **Infectious diseases of wild mammals**: Iowa State University Press Ames, IA, 2001.

de Caprariis, D.Dantas-Torres, F.Capelli, G.Mencke, N.Stanneck, D.Breitschwerdt, E.B. et al. Evolution of clinical, haematological and biochemical findings in young dogs naturally infected by vector-borne pathogens. **Veterinary Microbiology**, 149, n. 1, p. 206-212, 2011/04/21/ 2011.

de la Fuente, J.Atkinson, M.W.Naranjo, V.Fernández de Mera, I.G.Mangold, A.J.Keating, K.A. et al. Sequence analysis of the msp4 gene of Anaplasma ovis strains. **Veterinary Microbiology**, 119, n. 2–4, p. 375-381, 1/31/ 2007a.

de la Fuente, J.; Garcia-Garcia, J.C.; Blouin, E.F.; Kocan, K.M. Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen Anaplasma marginale to bovine erythrocytes and tick cells. **International Journal for Parasitology**, 31, n. 2, p. 145-153, 2// 2001a.

de la Fuente, J.; Garcia-Garcia, J.C.; Blouin, E.F.; Kocan, K.M. Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. **Veterinary Microbiology**, 91, n. 2-3, p. 265-283, 2003a.

de la Fuente, J.; Garcia-Garcia, J.C.; Blouin, E.F.; McEwen, B.R.; Clawson, D.; Kocan, K.M. Major surface protein 1a effects tick infection and transmission of *Anaplasma marginale*. **International Journal of Parasitology**, 31, n. 14, p. 1705-1714, Dec 2001b.

de la Fuente, J.; Hidalgo, Y.; Ochagavia, M.E.; Muzio, V.; Rodriguez, M.P. Analysis of enterovirus sequences recovered from the cerebrospinal fluid of patients with epidemic neuropathy. **Annals of Tropical Medicine & Parasitology**, 93, n. 2, p. 153-161, Mar 1999.

de la Fuente, J.Kocan, K.M.Blouin, E.F.Zivkovic, Z.Naranjo, V.Almazán, C. et al. Functional genomics and evolution of tick–*Anaplasma* interactions and vaccine development. **Veterinary Parasitology**, 167, n. 2-4, p. 175-186, 2010.

de la Fuente, J.Naranjo, V.Ruiz-Fons, F.Vicente, J.Estrada-Peña, A.Almazán, C. et al. Prevalence of tick-borne pathogens in ixodid ticks (Acari: Ixodidae) collected from European wild boar (*Sus scrofa*) and Iberian red deer (*Cervus elaphus hispanicus*) in central Spain. **European Journal of Wildlife Research**, 50, n. 4, p. 187-196, 2004/12/01 2004a.

de La Fuente, J.; Passos, L.M.; Van Den Bussche, R.A.; Ribeiro, M.F.; Facury-Filho, E.J.; Kocan, K.M. Genetic diversity and molecular phylogeny of *Anaplasma marginale* isolates from Minas Gerais, Brazil. **Veterinary Parasitology**, 121, n. 3-4, p. 307-316, May 26 2004b.

de la Fuente, J.Ruybal, P.Mtshali, M.S.Naranjo, V.Shuqing, L.Mangold, A.J. et al. Analysis of world strains of *Anaplasma marginale* using major surface protein 1a repeat sequences. **Veterinary Microbiology**, 119, n. 2–4, p. 382-390, 1/31/ 2007b.

de la Fuente, J.Thomas, E.J.G.Van den Bussche, R.A.Hamilton, R.G.Tanaka, E.E.Druhan, S.E. et al. Characterization of *Anaplasma marginale* isolated from north

American bison. **Applied and Environmental Microbiology**, 69, n. 8, p. 5001-5005, 2003b.

de la Fuente, J.Torina, A.Naranjo, V.Caracappa, S.Vicente, J.Mangold, A.J. et al. Genetic diversity of *Anaplasma marginale* strains from cattle farms in the province of Palermo, Sicily. **Journal of veterinary medicine. B, Infectious diseases and veterinary public health**, 52, n. 5, p. 226-229, 2005.

de la Fuente, J.Van Den Bussche, R.A.Garcia-Garcia, J.C.Rodriguez, S.D.Garcia, M.A.Guglielmone, A.A. et al. Phylogeography of New World isolates of *Anaplasma marginale* based on major surface protein sequences. **Veterinary Microbiology**, 88, n. 3, p. 275-285, 2002.

de la Fuente, J.; Van Den Bussche, R.A.; Kocan, K.M. Molecular phylogeny and biogeography of North American isolates of *Anaplasma marginale* (Rickettsiaceae: Ehrlichieae). **Veterinary Parasitology**, 97, n. 1, p. 65-76, 5/9/ 2001c.

de la Fuente, J.Vicente, J.n.Höfle, U.Ruiz-Fons, F.Fernández de Mera, I.G.Van Den Bussche, R.A. et al. Anaplasma infection in free-ranging Iberian red deer in the region of Castilla-La Mancha, Spain. **Veterinary Microbiology**, 100, n. 3–4, p. 163-173, 6/3/ 2004c.

Dikmans, G. The transmission of anaplasmosis. **American Journal of Veterinary Research**, 11, n. 38, 1950.

Douglas, A.D.; de Cassan, S.C.; Dicks, M.D.J.; Gilbert, S.C.; Hill, A.V.S.; Draper, S.J. Tailoring subunit vaccine immunogenicity: Maximizing antibody and T cell responses by using combinations of adenovirus, poxvirus and protein-adjuvant vaccines against Plasmodium falciparum MSP1. **Vaccine**, 28, n. 44, p. 7167-7178, 10/18/ 2010.

Drazenovich, N.; Foley, J.; Brown, R.N. Use of real-time quantitative PCR targeting the msp2 protein gene to identify cryptic Anaplasma phagocytophilum infections in wildlife and domestic animals. **Vector-Borne & Zoonotic Diseases**, 6, n. 1, p. 83-90, 2006.

Dumler, J.S.Barbet, A.F.Bekker, C.P.Dasch, G.A.Palmer, G.H.Ray, S.C. et al. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. **Internternationl Journal of Systematic and Evolucinnary Microbiology**, 51, n. Pt 6, p. 2145-2165, Nov 2001.

Dumler, J.S.Choi, K.-S.Garcia-Garcia, J.C.Barat, N.S.Scorpio, D.G.Garyu, J.W. et al. Human granulocytic anaplasmosis and Anaplasma phagocytophilum. **Emerging Infectious Diseases**, 11, n. 12, p. 1828-1834, 2005.

Eriks, I.S.; Stiller, D.; Palmer, G.H. Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. **Journal of Clinical Microbiology**, 31, n. 8, p. 2091-2096, 1993.

Estrada-Pena, A.; Naranjo, V.; Acevedo-Whitehouse, K.; Mangold, A.J.; Kocan, K.M.; de la Fuente, J. Phylogeographic analysis reveals association of tick- borne pathogen, *Anaplasma marginale*, MSP1a sequences with ecological traits affecting tick vector performance. **BMC Biology**, 7, 2009.

Ewing, S.A. Transmission of *Anaplasma marginale* by arthropods. *In*: Proceedings of the 7th National Anaplasmosis, 1981, Mississippi State University, MS, USA. p. 395–423.

Ewing, S.A., 1995, ***Ehrlichia canis* in tick cell culture**. Iowa State University Press. Chicago, EUA. In Proceedings of the 76th Conference of Research Workers in Animal Diseases, Chicago, USA, 13–14 November 1995.

Ferrolho, J.; Simpson, J.; Hawes, P.; Zweygarth, E.; Bell-Sakyi, L. Growth of *Ehrlichia canis*, the causative agent of canine monocytic ehrlichiosis, in vector and non-vector ixodid tick cell lines. **Ticks and Tick-borne Diseases**, 7, n. 4, p. 631-637, 6// 2016.

Figueroa, J.V.Alvarez, J.A.Ramos, J.A.Rojas, E.E.Santiago, C.Mosqueda, J.J. et al. Bovine babesiosis and anaplasmosis follow-up on cattle relocated in an endemic area for hemoparasitic diseases. **Annals of the New York Academy of Sciences**, 849, p. 1-10, Jun 29 1998.

Foil, L.D. Tabanids as vectors of disease agents. **Parasitology Today**, 5, n. 3, p. 88-96, Mar 1989.

French, D.M.; Brown, W.C.; Palmer, G.H. Emergence of Anaplasma marginale Antigenic Variants during Persistent Rickettsemia. **Infection and Immunity**, 67, n. 11, p. 5834, 1999.

French, D.M.; McElwain, T.F.; McGuire, T.C.; Palmer, G.H. Expression of *Anaplasma marginale* Major Surface Protein 2? Variants during Persistent Cyclic Rickettsemia. **Infection and Immunity**, 66, n. 3, p. 1200, 1998.

Futse, J.E.; Ueti, M.W.; Knowles, D.P., Jr.; Palmer, G.H. Transmission of *Anaplasma marginale* by *Boophilus microplus*: retention of vector competence in the absence of vector-pathogen interaction. **Journal of Clinical Microbiology**, 41, n. 8, p. 3829-3834, 2003.

Garcia-Garcia, J.C.de la Fuente, J.Kocan, K.M.Blouin, E.F.Halbur, T.Onet, V.C. et al. Mapping of B-cell epitopes in the N-terminal repeated peptides of *Anaplasma marginale*

major surface protein 1a and characterization of the humoral immune response of cattle immunized with recombinant and whole organism antigens. **Veterinary Immunology and Immunopathology**, 98, n. 3-4, p. 137-151, 2004.

García-Pérez, A.L.Oporto, B.Espí, A.Del Cerro, A.Barral, M.Povedano, I. et al. Anaplasmataceae in wild ungulates and carnivores in northern Spain. **Ticks Tick-borne Dis.**, 7, n. 2, p. 264, 2016.

Ge, N.L.; Kocan, K.M.; Blouin, E.F.; Murphy, G.L. Developmental studies of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in male *Dermacentor andersoni* (Acari: Ixodidae) infected as adult using nonradioactive in situ hybridization **Journal of Medical Entomology,**, 33, n. 6, p. 911–920, 1996.

Gilot, B.; Aubert, M. Ixodidae (Acari, Ixodoidea) parasite of wild Carnivora in French Alps and their fore countries. **Acarologia**, 26, n. 3, p. 215-233, 1985.

Goethert, H.K.; Telford, S.R. Enzootic Transmission of Anaplasma bovis in Nantucket Cottontail Rabbits. **Journal of Clinical Microbiology**, 41, n. 8, p. 3744, 2003.

Guglielmone, A.A. Epidemiology of babesiosis and anaplasmosis in South and Central America. **Veterinary Parasitology**, 57, n. 1–3, p. 109-119, 3// 1995.

Gulia-Nuss, M.Nuss, A.B.Meyer, J.M.Sonenshine, D.E.Roe, R.M.Waterhouse, R.M. et al. Genomic insights into the Ixodes scapularis tick vector of Lyme disease. **Nature communications**, 7, n. 1, p. 1-13, 2016.

Harrus, S.; Kass, P.H.; Klement, E.; Waner, T. Canine monocytic ehrlichiosis: a retrospective study of 100 cases, and an epidemiological investigation of prognostic indicators for the disease. **Veterinary Record**, 141, n. 14, p. 360, 1997.

Harrus, S.; Perlman-Avrahami, A.; Mumcuoglu, K.Y.; Morick, D.; Baneth, G. Molecular detection of *Rickettsia massiliae*, *Rickettsia sibirica mongolitimonae* and *Rickettsia conorii* israelensis in ticks from Israel. **Clinical Microbiology Infectction**, 17, n. 2, p. 176-180, 2011.

Harvey, J.W.; Simpson, C.F.; Gaskin, J.M. Cyclic thrombocytopenia induced by a *Rickettsia*-like agent in dogs. **J. Inf. Dis.**, 137, n. 2, p. 182-188, 1978.

Hosseini-Vasoukolaei, N.Oshaghi, M.A.Shayan, P.Vatandoost, H.Babamahmoudi, F.Yaghoobi-Ershadi, M.R. et al. Anaplasma Infection in Ticks, Livestock and Human in Ghaemshahr, Mazandaran Province, Iran. **Journal of arthropod-borne diseases**, 8, n. 2, p. 204-211, 2014.

Inokuma, H.; Raoult, D.; Brouqui, P. Detection of Ehrlichia platys DNA in brown dog ticks (Rhipicephalus sanguineus) in Okinawa Island, Japan. **Journal of Clinical Microbiology**, 38, n. 11, p. 4219-4221, 2000.

Jones, E.; Brock, W., 1966, **Bovine Anaplasmosis-Its Diagnosis Treatment and Control**. 1336-&. Disponível em: https://scholar.google.com.br/scholar?hl=pt-BR&q=Bovine+Anaplasmosis-Its+Diagnosis+Treatment+and+Control.

Jonsson, N.N.; Reid, S.W.J. Global climate change and vector borne diseases. **Veterinary Journal**, 160, n. 2, p. 87-89, 2000.

Kawahara, M.Rikihisa, Y.Lin, Q.Isogai, E.Tahara, K.Itagaki, A. et al. Novel genetic variants of Anaplasma phagocytophilum, Anaplasma bovis, Anaplasma centrale, and a novel Ehrlichia sp. in wild deer and ticks on two major islands in Japan. **Applied and Environmental Microbiology**, 72, n. 2, p. 1102-1109, Feb 2006.

Kessler, R.H.; Ristic, M. In vitro cultivation of *Anaplasma marginale*: invasion of and development of noninfected erythrocytes **American Journal of Veterinary Research**, 40, p. 1744–1776, 1979.

Khumalo, Z.T.H.; Brayton, K.A.; Collins, N.E.; Chaisi, M.E.; Quan, M.; Oosthuizen, M.C. Evidence confirming the phylogenetic position of Anaplasma centrale (ex Theiler 1911) Ristic and Kreier 1984. **International Journal of Systematic and Evolutionary Microbiology**, 68, n. 8, p. 2682-2691, 2018.

Kieser, S.; Eriks, I.; Palmer, G. Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle. **Infection and Immunity**, 58, n. 4, p. 1117-1119, 1990.

Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. **Journal of Molecular Evolution**, 16, n. 2, p. 111-120, Dec 1980.

KocanGoff, W.Stiller, D.Edwards, W.Ewing, S.Claypool, P. et al. Development of *Anaplasma marginale* in salivary glands of male *Dermacentor andersoni*. **American Journal of Veterinary Research**, 54, n. 1, p. 107-112, 1993.

Kocan, K.M. Development of *Anaplasma marginale* in ixodid ticks: coordinated development of a rickettsial organism and its tick host. . *In*: LTD, E. H. (Ed.). **Morphology, Physiology and Behavioral Ecology of Ticks.** England: Sauer, J.R., Hair, J.A., 1986. p. 472–505.

Kocan, K.M.Coetzee, J.F.Step, D.L.Blouin, E.Reppert, E.Simpson, K. et al. Current challenges in the diagnosis and control of bovine anaplasmosis. **Bovine Practitioner**, 46, n. 2, p. 67-77, 2012.

Kocan, K.M.; de la Fuente, J.; Blouin, E.F.; Coetzee, J.F.; Ewing, S.A. The natural history of *Anaplasma marginale*. **Veterinary Parasitology**, 167, n. 2–4, p. 95-107, 2/10/ 2010.

Kocan, K.M.; de la Fuente, J.; Blouin, E.F.; Garcia-Garcia, J.C. *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. **Parasitology**, 129 Suppl, p. S285-300, 2004 2004a.

Kocan, K.M.; De La Fuente, J.; Blouin, E.F.; Garcia-garcia, J.C.; de la Fuente, J.; Garcia-Garcia, J.C. *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. **Parasitology**, 129, p. 285, 2004b.

Kocan, K.M.; de la Fuente, J.; Cabezas-Cruz, A. The genus *Anaplasma*: new challenges after reclassification. **Revue Scientifique et Technique** 34, n. 2, p. 577-586, Aug 2015.

Kocan, K.M.; de la Fuente, J.; Guglielmone, A.A.; Melendez, R.D. Antigens and Alternatives for Control of *Anaplasma marginale* Infection in Cattle. **Clinical Microb. Rev.**, 16, n. 4, p. 698, 2003.

Kocan, K.M.Goff, W.L.Stiller, D.Claypool, P.L.Edwards, W.Ewing, S.A. et al. Persistence of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in male *Dermacentor andersoni* (Acari: Ixodidae) transferred successively from infected to susceptible cattle. . **Journal of Medical Entomology** 29, n. 4, p. 657–668, 1992.

Kuttler, K.L. *Anaplasma* infections in wild and domestic ruminants: a review. **Journal of Wildlife Diseases**, 20, n. 1, p. 12-20, Jan 1984.

Lallinger, G.; Zweygarth, E.; Bell-Sakyi, L.; Passos, L.M. Cold storage and cryopreservation of tick cell lines. **Parasit Vectors**, 3, n. 1, p. 37, Apr 13 2010.

Lew, A.E.; Bock, R.E.; Minchin, C.M.; Masaka, S. A msp1 alpha polymerase chain reaction assay for specific detection and differentiation of *Anaplasma marginale* isolates. **Veterinary Microbiology**, 86, n. 4, p. 325-335, 2002.

Li, H.-t.Sun, L.-s.Chen, Z.-m.Hu, J.-s.Ye, C.-d.Jia, K. et al. Detection of Anaplasma platys in dogs using real-time loop-mediated isothermal amplification. **The Veterinary Journal**, 199, n. 3, p. 468-470, 2014/03/01/ 2014.

Li, H.Zheng, Y.-C.Ma, L.Jia, N.Jiang, B.-G.Jiang, R.-R. et al. Human infection with a novel tick-borne Anaplasma species in China: a surveillance study. **The Lancet Infectious Diseases**, 15, n. 6, p. 663-670, 2015/06/01/ 2015a.

Li, J.; Kelly, P.; Zhang, J.; Xu, C.; Wang, C. Development of a pan-Babesia FRET-qPCR and a survey of livestock from five Caribbean islands. **BMC Veterinary Research**, 11, n. 1, p. 246, 2015b. journal article.

Li, Y.Yang, J.Chen, Z.Qin, G.Li, Y.Li, Q. et al. Anaplasma infection of Bactrian camels (Camelus bactrianus) and ticks in Xinjiang, China. **Parasites & vectors**, 8, n. 1, p. 313, 2015c.

Lindh, J.M.; Terenius, O.; Faye, I. 16S rRNA gene-based identification of midgut bacteria from field-caught Anopheles gambiae sensu lato and A. funestus mosquitoes reveals new species related to known insect symbionts. **Applied and Environmental Microbiology**, 71, n. 11, p. 7217-7223, 2005.

Liu, Z.Ma, M.Wang, Z.Wang, J.Peng, Y.Li, Y. et al. Molecular Survey and Genetic Identification of Anaplasma Species in Goats from Central and Southern China. **Applied and Environmental Microbiology**, 78, n. 2, p. 464, 2012.

Losos, G.J. **Infectious tropical diseases of domestic animals**. Longman Scientific & Technical, 1986. 0582404088.

Machado, R.Z.; Silva, J.B.d.; André, M.R.; Gonçalves, L.R.; Matos, C.A.; Obregón, D. Outbreak of anaplasmosis associated with the presence of different *Anaplasma marginale* strains in dairy cattle in the states of São Paulo and Goiás, Brazil. **Rev. Brasil. Parasitol. Vet.**, 24, n. 4, p. 438-446, 2015.

Maggi, R.G.; Mascarelli, P.E.; Havenga, L.N.; Naidoo, V.; Breitschwerdt, E.B. Co-infection with *Anaplasma platys*, *Bartonella henselae* and Candidatus *Mycoplasma haematoparvum* in a veterinarian. **Parasit Vect**, 6, n. 1, p. 103, 2013.

Martin, A.R.; Brown, G.K.; Hugh Dunstan, R.; Roberts, T.K. Anaplasma platys: an improved PCR for its detection in dogs. **Experimental Parasitology**, 109, n. 3, p. 176-180, 2005/03/01/ 2005.

Massey, S.E.; Churbanov, A.; Rastogi, S.; Liberles, D.A. Characterizing positive and negative selection and their phylogenetic effects. **Gene**, 418, n. 1, p. 22-26, 2008/07/15/ 2008.

Masuzawa, T.Uchishima, Y.Fukui, T.Okamoto, Y.Pan, M.-J.Kadosaka, T. et al. Detection of Anaplasma phagocytophilum and Anaplasma bovis in Small Wild Mammals from Taichung and Kinmen Island, Taiwan. **Japanese Journal of Infectious Diseases**, 67, n. 2, p. 111-114, 2014.

Mazzola, V.; Amerault, T.E.; Roby, T.O. Survival of *Anaplasma marginale* in Aedes albopictus cells. . **American Journal of Veterinary Research**, 37, p. 987–989, 1976.

McGarey, D.J.; Allred, D.R. Characterization of hemagglutinating components on the *Anaplasma marginale* initial body surface and identification of possible adhesins. **Infection and Immunity**, 62, n. 10, p. 4587, 1994a.

McGarey, D.J.; Barbet, A.F.; Palmer, G.H.; McGuire, T.C.; Allred, D.R. Putative adhesins of *Anaplasma marginale:* major surface polypeptides 1a and 1b. **Infec. Immun.**, 62, n. 10, p. 4594-4601, 1994b.

Melo, A.L.T.Witter, R.Martins, T.F.Pacheco, T.A.Alves, A.S.Chitarra, C.S. et al. A survey of tick-borne pathogens in dogs and their ticks in the Pantanal biome, Brazil. **Medical and Veterinary Entomology**, 30, n. 1, p. 112-116, 2016/03/01 2016.

Munderloh, U.G.; Blouin, E.F.; Kocan, K.M.; Ge, N.L.; Edwards, W.L.; Kurtti, T.J. Establishment of the tick (Acari:Ixodidae)-borne cattle pathogen *Anaplasma marginale* (Rickettsiales:Anaplasmataceae) in tick cell culture. **Journal of Meducak Entomology**, 33, n. 4, p. 656-664, Jul 1996.

Munderloh, U.G.; Liu, Y.; Wang, M.; Chen, C.; Kurtti, T.J. Establishment, maintenance and description of cell lines from the tick *Ixodes scapularis*. **J. Parasit.**, 80, n. 4, p. 533-543, Aug 1994.

Munderloh, U.G.Lynch, M.J.Herron, M.J.Palmer, A.T.Kurtti, T.J.Nelson, R.D. et al. Infection of endothelial cells with *Anaplasma marginale* and *A. phagocytophilum*. **Veterinary Microbiology**, 101, n. 1, p. 53-64, 2004.

Murphy, G.L.; Ewing, S.; Whitworth, L.C.; Fox, J.C.; Kocan, A.A. A molecular and serologic survey of *Ehrlichia canis*, *E. chaffeensis*, and *E. ewingii* in dogs and ticks from Oklahoma. ***Vet . Parasitol.***, 79, n. 4, p. 325-339, 1998.

Mutshembele, A.M.; Cabezas-Cruz, A.; Mtshali, M.S.; Thekisoe, O.M.; Galindo, R.C.; de la Fuente, J. Epidemiology and evolution of the genetic variability of *Anaplasma marginale* in South Africa. **Ticks and tick-borne diseases**, 5, n. 6, p. 624-631, 2014.

Nuttall, P. Displaced tick-parasite interactions at the host interface. **Parasitology**, 116, n. S1, p. S65-S72, 1998.

Oberle, S.M.; Barbet, A.F. Derivation of the complete msp4 gene sequence of *Anaplasma marginale* without cloning. **Gene**, 136, n. 1-2, p. 291-294, 1993.

Palmer, G.H.; Brayton, K.A. Antigenic variation and transmission fitness as drivers of bacterial strain structure. **Cellular Microbiology**, 15, n. 12, p. 1969-1975, 2013.

Palmer, G.H.; Eid, G.; Barbet, A.F.; McGuire, T.C.; McElwain, T.F. The immunoprotective *Anaplasma marginale* major surface protein 2 is encoded by a polymorphic multigene family. **Infection and Immunity**, 62, n. 9, p. 3808-3816, 1994.

Palmer, G.H.; Rurangirwa, F.R.; McElwain, T.F. Strain composition of the Ehrlichia *Anaplasma marginale* within persistently infected cattle, a mammalian reservoir for tick transmission. **J. Clin. Microb.**, 39, n. 2, p. 631-635, 2001.

Palmer, G.H.; Waghela, S.D.; Barbet, A.F.; Davis, W.C.; McGuire, T.C. Characterization of a neutralization-sensitive epitope on the Am 105 surface protein of *Anaplasma marginale*. **International Journal of Parasitology**, 17, n. 7, p. 1279-1285, 1987.

Palomar, A.M.Portillo, A.SantibÁÑEz, P.Mazuelas, D.Roncero, L.GarcÍA-ÁLvarez, L. et al. Detection of tick-borne Anaplasma bovis, Anaplasma phagocytophilum and Anaplasma centrale in Spain. **Medical and Veterinary Entomology**, 29, n. 3, p. 349-353, 2015/09/01 2015.

Passos, L.M.F. In vitro cultivation of *Anaplasma marginale* and *A. phagocytophilum* in tick cell lines: a review. **Revista Brasileira de Parasitologia Veterinária**, 21, n. 2, p. 81-86, Apr-Jun 2012.

Peng, Y.Wang, K.Zhao, S.Yan, Y.Wang, H.Jing, J. et al. Detection and phylogenetic characterization of *Anaplasma capra*: an emerging pathogen in sheep and goats in China. **Front. cel. Inf. Microb.**, 8, p. 283, 2018.

Pereira, A.Parreira, R.Nunes, M.Casadinho, A.Vieira, M.L.Campino, L. et al. Molecular detection of tick-borne bacteria and protozoa in cervids and wild boars from Portugal. **Parasites & vectors**, 9, n. 1, p. 251, 2016.

Pohl, A.E.Cabezas-Cruz, A.Ribeiro, M.F.B.da Silveira, J.A.G.Silaghi, C.Pfister, K. et al. Detection of genetic diversity of *Anaplasma marginal*e isolates in Minas Gerais, Brazil. **Rev. Bra.. Parasitol. Vet.**, 22, n. 1, p. 129-135, 2013.

Popov, V.Han, V.C.Chen, S.-M.Dumler, J.Feng, H.-M.Andreadis, T. et al. Ultrastructural differentiation of the genogroups in the genus *Ehrlichia*. **Journal of Medical Microbiology**, 47, n. 3, p. 235-251, 1998.

Potgieter, F.; Van Rensburg, L. The persistence of colostral *Anaplasma* antibodies and incidence of in utero transmission of *Anaplasma* infections in calves under laboratory conditions. **Onderst. J.Vet. Res.**, 54, p. 557-560, 1987.

Potgieter, F.T.; Van Rensburg, L. The effect of incubation and prefeeding of infected *Rhipicephalus simus* nymphae and adults on the transmission of *Anaplasma marginale*. **Onderstepoort Journal of Veterinary Research**, 49, n. 2, p. 99-101, Jun 1982.

Qurollo, B.A.Chandrashekar, R.Hegarty, B.C.Beall, M.J.Stillman, B.A.Liu, J. et al. A serological survey of tick-borne pathogens in dogs in North America and the Caribbean as assessed by Anaplasma phagocytophilum, A. platys, Ehrlichia canis, E. chaffeensis, E. ewingii, and Borrelia burgdorferi species-specific peptides. **Infection Ecology & Epidemiology**, 4, n. 1, p. 24699, 2014/01/01 2014.

Rådström, P.; Knutsson, R.; Wolffs, P.; Lövenklev, M.; Löfström, C. Pre-PCR processing. **Molecular Biotechnology**, 26, n. 2, p. 133-146, 2004.

Ramos, R.Latrofa, M.S.Giannelli, A.Lacasella, V.Campbell, B.Dantas-Torres, F. et al. Detection of Anaplasma platys in dogs and Rhipicephalus sanguineus group ticks by a quantitative real-time PCR. **Veterinary Parasitology**, 205, n. 1-2, p. 285-288, 2014.

Rar, V.; Golovljova, I. Anaplasma, Ehrlichia, and "Candidatus Neoehrlichia" bacteria: Pathogenicity, biodiversity, and molecular genetic characteristics, a review. **Infection, Genetics and Evolution**, 11, n. 8, p. 1842-1861, 2011/12/01/ 2011.

Ristic, M.; Carson, C. Methods of immunoprophylaxis against bovine anaplasmosis with emphasis on use of the attenuated *Anaplasma marginale* vaccine. *In*: **Immunity to blood parasites of animals and man**: Springer, 1977. p. 151-188.

Rossen, L.; Nørskov, P.; Holmstrøm, K.; Rasmussen, O.F. Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. **International Journal of Food Microbiology**, 17, n. 1, p. 37-45, 1992.

Roy, B.Krücken, J.Ahmed, J.Majumder, S.Baumann, M.Clausen, P.H. et al. Molecular identification of tick-borne pathogens infecting cattle in Mymensingh district of Bangladesh reveals emerging species of Anaplasma and Babesia. **Transboundary and emerging diseases**, 65, n. 2, p. e231-e242, 2018.

Ruiz, P.M.G.; Passos, L.M.F.; Barbosa Ribeiro, M.F. Lack of infectivity of a Brazilian Anaplasma marginale isolate for Boophilus microplus ticks. **Veterinary Parasitology**, 128, n. 3, p. 325-331, 2005/03/31/ 2005.

Rymaszewska, A.; Grenda, S. Bacteria of the genus *Anaplasma*–characteristics of *Anaplasma* and their vectors: a review. **Vet.y Med. J.**, 53, n. 11, p. 573-584, 2008.

Said, M.B.; Belkahia, H.; Messadi, L. Anaplasma spp. in North Africa: a review on molecular epidemiology, associated risk factors and genetic characteristics. **Ticks and Tick-borne Diseases**, 9, n. 3, p. 543-555, 2018.

Sainz, A.; Amusategui, I.; Tesouro, M.A. Ehrlichia Platys Infection and Disease in Dogs in Spain. **Journal of Veterinary Diagnostic Investigation**, 11, n. 4, p. 382-384, 1999/07/01 1999.

Salabarria, F.; Pino, R. Transmisión vertical de *Anaplasma marginale* en bovinos afectados durante el período final de la gestación. **Revista Cubana de Ciencias Veterinarias**, 19, n. 3, p. 179-182, 1988.

Salmon, D.Harbaugh, W.H.Law, J.Dickson, W.Trumbower, M.R.Smith, T. et al. **Special report on diseases of cattle and on cattle feeding**. US Government Printing Office, 1896.

Sanogo, Y.; Inokuma, H.; Parola, P.; Brouqui, P.; Davoust, B.; Camicas, J. First evidence of Anaplasma platys in Rhipicephalus sanguineus (Acari: Ixodida) collected from dogs in Africa. **Onderstepoort Journal of Veterinary Research**, 70, p. 205-2012, 2003.

Santos, C.; Carvalho, C. First report of *Anaplasma bovis* (Donatien and Lestoquard, 1936) Dumler et al. (2001) at micro region of Campos dos Goytacazes, State of Rio de Janeiro, Brazil. **Revista Brasileira de Parasitologia Veterinaria**, 15, p. 126-127, 07/01 2006.

Santos, P.N.; Almeida Valim, J.R.; Matos, P.C.M.; Silva, J.B.; Fonseca, A.H. Molecular characterization of the msp1 α AmRio1 strain of *Anaplasma marginale* in calves and experimentally infected ticks. **Veterinary Parasitology: Regional Studies and Reports**, 16, p. 100268, 2019.

Sasaki, H.Ichikawa, Y.Sakata, Y.Endo, Y.Nishigaki, K.Matsumoto, K. et al. Molecular survey of Rickettsia, Ehrlichia, and Anaplasma infection of domestic cats in Japan. **Ticks and Tick-borne Diseases**, 3, n. 5, p. 308-311, 2012/12/01/ 2012.

Sato, M.Nishizawa, I.Fujihara, M.Nishimura, T.Matsubara, K.Harasawa, R. et al. Phylogenetic Analysis of the 16S rRNA Gene of Anaplasma Species Detected from Japanese Serows (Capricornis crispus). **Journal of Veterinary Medical Science**, 71, n. 12, p. 1677-1679, 2009.

Schrader, C.; Schielke, A.; Ellerbroek, L.; Johne, R. PCR inhibitors–occurrence, properties and removal. **Journal of Applied Microbiology**, 113, n. 5, p. 1014-1026, 2012.

Scoles, G.A.; Broce, A.B.; Lysyk, T.J.; Palmer, G.H. Relative efficiency of biological transmission of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) by *Dermacentor andersoni* (Acari: Ixodidae) compared with mechanical transmission by *Stomoxys calcitrans* (Diptera: Muscidae). **Journal of Medical Entomology**, 42, n. 4, p. 668-675, 2005.

Scoles, G.A.; Goff, W.L.; Lysyk, T.J.; Lewis, G.S.; Knowles, D.P. Validation of an *Anaplasma marginale* cELISA for use in the diagnosis of *A. ovis* infections in domestic sheep and Anaplasma spp. in wild ungulates. **Veterinary Microbiology**, 130, n. 1, p. 184-190, 2008/07/27/ 2008.

Shkap, V.Leibovitz, B.Krigel, Y.Molad, T.Fish, L.Mazuz, M. et al. Concomitant infection of cattle with the vaccine strain Anaplasma marginale ss centrale and field strains of A. marginale. **Veterinary Microbiology**, 130, n. 3, p. 277-284, 2008/08/25/ 2008.

Shompole, S.; Waghela, S.D.; Rurangirwa, F.R.; McGuire, T. Cloned DNA probes identify Anaplasma ovis in goats and reveal a high prevalence of infection. **Journal of Clinical Microbiology**, 27, n. 12, p. 2730-2735, 1989.

Silva, J.B.; Goncalves, L.R.; Varani Ade, M.; Andre, M.R.; Machado, R.Z. Genetic diversity and molecular phylogeny of *Anaplasma marginale* studied longitudinally under natural transmission conditions in Rio de Janeiro, Brazil. **Ticks Tick Borne Dis.**, 6, n. 4, p. 499-507, Jun 2015.

Simpson, R.M.; Gaunt, S.D.; Hair, J.A.; Kocan, K.M.; Henk, W.G.; Casey, H.W. Evaluation of *Rhipicephalus sanguineus* as a potential biologic vector of *Ehrlichia platys*. **American Journal of Veterinary Research**, 52, n. 9, p. 1537-1541, 1991.

Singh, H.; Jyoti; Haque, M.; Singh, N.K.; Rath, S.S. Molecular detection of *Anaplasma marginale* infection in carrier cattle. **Ticks Tick-borne Dis**, 3, n. 1, p. 55-58, 2// 2012.

Smith, T.H.; Kilborne, F.L. Investigation into the nature causation and prevision of Texas or southern cattle fever. **Department of Agricultural. Bulletin of Animal Industry**, 1, p. 177-304, 1893.

Splitter, E.; Anthony, H.; Twiehaus, M. Anaplasma ovis *in the United States; experimental* studies with sheep and goats. **American Journal of Veterinary Research**, 17, n. 64, p. 487, 1956.

Sreekumar, C.; Anandan, R.; Balasundaram, S. Incidence of Ehrlichia bovis infection in Tamil Nadu. **Indian Journal of Animal Sciences**, 69, n. 12, p. 1005-1007, 1999.

Stich, R.W.; Kocan, K.M.; Palmer, G.H.; Ewing, S.A.; Hair, J.A.; Barron, S.J. Transstadial and attempted transovarial transmission of Anaplasma marginale by Dermacentor variabilis. **American Journal of Veterinary Research**, 50, n. 8, p. 1377–1380, 1989.

Stuen, S. Anaplasma Phagocytophilum - the Most Widespread Tick-Borne Infection in Animals in Europe. **Veterinary Research Communications**, 31, n. 1, p. 79-84, 2007/08/01 2007.

Stuen, S.; Bergström, K.; Palmér, E. Reduced Weight Gain Due to Subclinical Anaplasma phagocytophilum (Formerly Ehrlichia phagocytophila) Infection. **Experimental & Applied Acarology**, 28, n. 1, p. 209-215, 2002/05/01 2002.

Tamí, I.; Jordán, L. Identificación de mórulas de *Ehrlichia* en plaquetas de sangre humana en Venezuela. **Antibioticos e infecction**, 10, p. 123-128, 2002.

Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: molecular evolutionary genetics analysis version 6.0. **Molecular Biology and Evolution**, 30, n. 12, p. 2725-2729, 2013.

Tatchell, R.; Moorhouse, D. Neutrophils: their role in the formation of a tick feeding lesion. **Science**, 167, n. 3920, p. 1002-1003, 1970.

Tate, C.M.Howerth, E.W.Mead, D.G.Dugan, V.G.Luttrell, M.P.Sahora, A.I. et al. *Anaplasma odocoilei* sp. nov.(family Anaplasmataceae) from white-tailed deer (*Odocoileus virginianus*). **Ticks and tick-borne diseases**, 4, n. 1-2, p. 110-119, 2013.

Theiler, A. *Anaplasma marginale* (gen. and spec. nov.): a protozoon of cattle; a cause of the called gallsickness. **Transvaal Med. J.**, p. 110-111, 1910.

Theiler, A. **Further investigations into anaplasmosis of South African cattle**. In: 1st Report of the Director of Veterinary Research, Department of Agriculture of the Union of South Africa., p. 7-46. 1911.

Torioni de Echaide, S.; Knowles, D.P.; McGuire, T.C.; Palmer, G.H.; Suarez, C.E.; McElwain, T.F. Detection of Cattle Naturally Infected with *Anaplasma marginale* in a Region of Endemicity by Nested PCR and a Competitive Enzyme-Linked Immunosorbent Assay Using Recombinant Major Surface Protein 5. **J. Cli. Microbiol.**, 36, n. 3, p. 777, 1998.

Trotta, M.; Fogliazza, A.; Furlanello, T.; Solano-Gallego, L. A molecular and serological study of exposure to tick-borne pathogens in sick dogs from Italy. **Clinical Microbiology and Infection**, 15, p. 62-63, 2009.

Uilenberg, G. Other ehrlichioses of ruminants. **Rickettsial and chlamydial diseases of domestic animals. Pergamon Press, Oxford, United Kingdom**, p. 269-279, 1993.

Vargas, M.; Soberanes Céspedes, N.; Fragoso Sánchez, H.; Martins, J.R.; Cordovés Céspedes, C.O. Avaliação in vitro de uma cepa de campo de *Boophilus microplus* (Acari : Ixodidae) resistente à amitraz. **Ciencia Rural**, 33, p. 737-742, 2003.

Viseshakul, N.; Kamper, S.; Bowie, M.V.; Barbet, A.F. Sequence and expression analysis of a surface antigen gene family of the rickettsia Anaplasma marginale. **Gene**, 253, n. 1, p. 45-53, 7/25/ 2000.

Woldehiwet, Z. The effects of tick-borne fever on some functions of polymorphonuclear cells of sheep. **Journal of Comparative Pathology**, 97, n. 4, p. 481-485, 1987/07/01/ 1987.

Woldehiwet, Z. The natural history of Anaplasma phagocytophilum. **Veterinary Parasitology**, 167, n. 2, p. 108-122, 2010/02/10/ 2010.

Woldehiwet, Z.; Scott, G.R. Stages in the development of Cytoecetes phagocytophila, the causative agent of tick-borne fever. **Journal of Comparative Pathology**, 92, n. 3, p. 469-474, 1982/07/01/ 1982.

Woody, B.J.; Hoskins, J.D. Ehrlichial diseases of dogs. **Veterinary Clinics of North America: Small Animal Practice**, 21, n. 1, p. 75-98, 1991.

Yang, J.Han, R.Niu, Q.Liu, Z.Guan, G.Liu, G. et al. Occurrence of four Anaplasma species with veterinary and public health significance in sheep, northwestern China. **Ticks and Tick-borne Diseases**, 9, n. 1, p. 82-85, 2018/01/01/ 2018.

Yang, J.Liu, Z.Niu, Q.Liu, J.Han, R.Liu, G. et al. Molecular survey and characterization of a novel Anaplasma species closely related to Anaplasma capra in ticks, northwestern China. **Parasites & Vectors**, 9, n. 1, p. 603, 2016/11/25 2016.

Yoshimoto, K.Matsuyama, Y.Matsuda, H.Sakamoto, L.Matsumoto, K.Yokoyama, N. et al. Detection of Anaplasma bovis and Anaplasma phagocytophilum DNA from Haemaphysalis megaspinosa in Hokkaido, Japan. **Veterinary Parasitology**, 168, n. 1, p. 170-172, 2010/02/26/ 2010.

Zabel, T.A.; Agusto, F.B. Transmission Dynamics of Bovine Anaplasmosis in a Cattle Herd. **Interdiscip Perspect of Infectious Diseases**, 2018, p. 4373981, 2018.

Zaugg, J. Bovine anaplasmosis: transplacental transmission as it relates to stage of gestation. **American Journal of Veterinary Research**, 46, n. 3, p. 570-572, 1985.

Zaugg, J.; Stiller, D.; Coan, M.; Lincoln, S. Transmission of *Anaplasma marginale* Theiler by males of *Dermacentor andersoni* Stiles fed on an Idaho field-infected, chronic carrier cow. **American Journal of Veterinary Research**, 47, n. 10, p. 2269-2271, 1986.

Zaugg, J.L.; Goff, W.L.; Foreyt, W.; Hunter, D.L. Susceptibility of elk (*Cervus elaphus*) to experimental infection with *Anaplasma marginale* and *A. ovis*. **Journal of Wildlife Diseases**, 32, n. 1, p. 62-66, 1996.

Zaugg, J.L.; Kuttler, K.L. Bovines anaplasmosis: in utero transmission and the immunologic significance of ingested colostral antibodies **American Journal of Veterinary Research** 45, n. 3, p. 570-572, 1984.

Zobba, R.Anfossi, A.G.Parpaglia, M.L.P.Dore, G.M.Chessa, B.Spezzigu, A. et al. Molecular investigation and phylogeny of Anaplasma spp. in Mediterranean ruminants reveal the presence of neutrophil-tropic strains closely related to *A. platys*. **Applied and Environmental Microbiology**, 80, n. 1, p. 271-280, 2014.

Zweygarth, E.Cabezas-Cruz, A.Josemans, A.I.Oosthuizen, M.C.Matjila, P.T.Lis, K. et al. In vitro culture and structural differences in the major immunoreactive protein gp36 of geographically distant Ehrlichia canis isolates. **Ticks and tick-borne diseases**, 5, n. 4, p. 423-431, 2014a.

Zweygarth, E.; Josemans, A. l-cysteine replaces microaerophilous culture conditions for the in vitro initiation of Theileria equi. **Parasitology Research**, 113, n. 1, p. 433-435, 2014b.

Zweygarth, E.Josemans, A.I.Spickett, A.M.Steyn, H.C.Putterill, J.Troskie, P.C. et al. In vitro cultivation of a south African isolate of an Anaplasma sp. in tick cell cultures. **The Onderstepoort journal of veterinary research**, 73, n. 4, p. 251, 2006a.

Zweygarth, E.Josemans, A.I.Spickett, A.M.Steyn, H.C.Putterill, J.Troskie, P.C. et al. In vitro cultivation of a south African isolate of an *Anaplasma* sp. in tick cell cultures. **Onderstepoort Journal of Veterinary Research**, 73, n. 4, p. 251-255, Dec 2006b.

Zweygarth, E.Schöl, H.Lis, K.Cabezas Cruz, A.Thiel, C.Silaghi, C. et al. In vitro Culture of a Novel Genotype of E hrlichia sp. from B razil. **Transboundary and emerging diseases**, 60, p. 86-92, 2013.

7 GENERAL CONCLUSIONS

This study provided results that filled the gaps regarding the diversity of the MSP1a protein in an ecoregion with greater selective pressure, involving climate conditions of tropical countries and *Rhipicephalus microplus* as a vector. At the same time, further studies are suggested regarding the life cycle of *A. marginale*, since it was possible to isolate it from a purified buffy coat.

In addition, this study isolated and propagated a new genotype of *Anaplasma* sp. which can contribute for a better understanding of the evolutionary relationships among species of the genus *Anaplasma*.

ANNEXES

Annex I - Technical opinion Consubstantiated by the Ethics Committee on the Use of Animals at UFRRJ, approving the final report of the proposed project.

Annex II - Editorial Certificate of the thesis proofreading and editing by Cambridge Proofreading LLC.

Annex I – Technical opinion Consubstantiated by the Ethics Committee on the Use of Animals at UFRRJ, approving the final report of the proposed project.

UFRRJ
Universidade Federal Rural do Rio de Janeiro

Comissão de Ética no Uso de Animais
Instituto de Veterinária

CEUA

Seropédica, 07 de maio de 2020
CEUA N 2134171215

Ilmo(a). Sr(a).
Responsável: Adivaldo Henrique Da Fonseca
Área: Doenças Parasitárias

Título da proposta: "Diversidade da MSP1a e variabilidade da MSP2 nas cepas AmRio 1 e AmRio 2 de Anaplasma marginale em bezerros e carrapatos experimentalmente infectados".

Parecer Consubstanciado da Comissão de Ética no Uso de Animais UFRRJ (ID 001155)

A Comissão de Ética no Uso de Animais da Instituto de Veterinária da Universidade Federal Rural do Rio de Janeiro, no cumprimento das suas atribuições, analisou e **APROVOU** o Relatório Final (versão de 09/março/2020) da proposta acima referenciada.

Resumo apresentado pelo pesquisador: "1. Os objetivos propostos foram todos cumpridos? Resp: Sim Os resultados obtidos propiciaram a criação de novas atividades? Resp: Os resultados geraram hipóteses que podem continuar a ser exploradas em trabalhos futuros 2. O N amostral proposto inicialmente foi suficiente? Resp: Sim, pois este trabalho é de natureza qualitativa 3. Houve perdas? se sim, quantas? Resp: Não 4. Ocorreu algum evento adverso durante a condução das atividades? Resp: Não 5. As atividades foram gravadas e/ou transmitidas de alguma forma? Resp: Este projeto fazia parte da tese de uma discente e foi concluído 6. Qual foi o número total de alunos participantes? Resp: 4 alunos".

Comentário da CEUA: "Relatório aprovado.".

Fabio B. Scott

Prof. Dr. Fabio Barbour Scott
Coordenador da Comissão de Ética no Uso de Animais
Instituto de Veterinária da Universidade Federal Rural do Rio de Janeiro

Carlos Alexandre Rey Matias
Vice-Coordenador da Comissão de Ética no Uso de Animais
Instituto de Veterinária da Universidade Federal Rural do Rio de Janeiro

Annex II - Editorial Certificate of the thesis proofreading and editing by Cambridge Proofreading LLC.

EDITORIAL CERTIFICATE

Authors:	Document title:	Date Issued:
PhD Thesis - Chapter 01 - Priscilla Nunes dos Santos	Diversity of the Anaplasma marginale msp1α gene and isolation and propagation of a novel genotype of Anaplasma sp. in IDE8 cells	6 May 2020

Cambridge Proofreading LLC

This document certifies that the above manuscript was proofread and edited by Cambridge Proofreading LLC.

This document certifies that the above manuscript was proofread and edited by Cambridge Proofreading Worldwide LLC. The document was edited for proper English language, grammar, punctuation, spelling, and overall style by one or more of our academic editors. The editor endeavoured to ensure that the author's intended meaning was not altered during the review. All amendments were tracked with the Microsoft Word 'Track Changes' feature. Therefore, the authors had the option to reject or accept each change individually.

Kind regards,
Cambridge Proofreading

Cambridge Proofreading Worldwide LLC is a registered company headquartered in Chicago, Illinois, USA with a global presence. All of our editors are native speakers from USA and the UK. Our Certificate of Good Standing can be found in the Illinois state business database by searching our name here.