IAGO CARVALHO BARBOSA

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Londrina
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Dissertação apresentada ao Programa de Pós-Graduação em Ciência Animal da Universidade Estadual de Londrina como requisito parcial para a obtenção do título de Mestre.

Orientador: Rafael Felipe da Costa Vieira

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Ficha de identificação da obra elaborada pelo autor, através do Programa de Geração Automática do Sistema de Bibliotecas da UEL

Barbosa, Iago Carvalho.
Anaplasma marginale in a Multi-Species Grazing Farm in the State of Paraiba, Northeastern Brazil / Iago Carvalho Barbosa. - Londrina, 2019.
50 f.

Orientador: Rafael Felipe da Costa Vieira.
Dissertação (Mestrado em Ciência Animal) - Universidade Estadual de Londrina, Centro de Ciências Agrárias, , 2019.
Inclui bibliografia.

1. Anaplasma marginale - Tese. 2. PCR e sorologia - Tese. 3. Genótipo - Tese. 4. MSP - Tese. I. Vieira, Rafael Felipe da Costa. II. Universidade Estadual de Londrina. Centro de Ciências Agrárias. . III. Título.

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Londrina, 15 de fevereiro de 2019.

Dedico este trabalho, aos meus pais,
Linaldo Laerson Barbosa e Kyvânia Carvalho Barbosa,
protetores e incentivadores da minha ciência.
Ainda as minha avós,
Josefa Dorziat Quirino Barbosa e Rita Silva de Carvalho,
de quem guardo imensa saudade e carinho.

AGRADECIMENTOS

Mais uma etapa concluída e com ela um grande sentimento de gratidão a todos que participaram de minha trajetória. Impossível falar em trajetória de vida e esquecer das pessoas que mais deram suporte e incentivo para sempre manter vívida a chama da ciência e a fome por novos conhecimentos. Aos meus pais, Linaldo e Kyvânia Barbosa, além de agradecer por minha vida, educação, formação e caráter, agradeço pelo carinho, paciência e amor dados em imensidão e sem pestanejar.

Ao meu professor, mentor e orientador, Rafael Vieira, agradeço pela caminhada de longos sete anos. Agradeço pela confiança, pelas responsabilidades e pela amizade, uma vez que me cresceram não só como profissional, mas como pessoa. Que nossa parceria se postergue e traga muitos frutos ainda. A sua esposa e parceira de trabalho, Thállitha Vieira, agradeço pelas cobranças, mas sempre realizadas de maneira bem-humorada e contente, alegrando nossos dias mesmo nos momentos mais complicados.

Agradeço a todos que direta ou indiretamente me ajudaram nessa caminhada. Parceiros de bancada, escrita e leitura da UFPR e UEL; amigos que fizeram de Londrina um lugar mais aconchegante e de mais fácil vivência longe de casa; professores de graduação, UFPB e NCSU, e pós-graduação, UEL, que incentivaram e alimentaram minha mente com conhecimentos tantas vezes importantes; meus amigos de longa data, que mesmo longe nunca me esqueceram.

O último parágrafo se destina não às últimas pessoas que pensei, mas àquelas que guardo um sentimento de carinho e saudade imensurável. Aos meus irmãos, Igor e Iann Carvalho Barbosa, um agradecimento todo especial pelos nossos hematomas, pertences quebrados e discussões, mas também pela nossa confidência, lealdade e parceria que hoje aqui não se acabam, mas se fortalecem a cada dia. Às minhas avós, Rita Carvalho e Josefa Dorziat, por quem guardo imenso carinho e saudade, agradeço pelo amor incondicional, conversas e por fazerem de suas casas uma extensão da minha. A saudade é imensa e me alegra saber que em meu retorno, poderei ver o sorriso incompleto da minha querida gordinha de cabelos brancos e poderei te abraçar e me sentir acolhido e querido. De minha outra gordinha, o sentimento de saudade se exacerba, mesmo não guardando sentimento de tristeza, lembrar dos nossos momentos de risadas, músicas e conversas de acasalamento de mosquito à atracamento de navio me traz alegria imensa.

Agradeço pela vida e mente pensante que me foram concedidas por qualquer força ou ainda de maneira aleatória.

BARBOSA, Iago Carvalho. ***Anaplasma marginale*** **em uma Fazenda de Criação Consorciada no Estado da Paraíba, Nordeste do Brasil.** 2019. 50 f. Dissertação (Mestrado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2019.

RESUMO

Anaplasma marginale, agente etiológico da anaplasmose bovina, pertence à família Anaplasmataceae e ordem Rickettsiales. Tal organismo aparece como corpos intraeritrocíticos densos, arredondados, com aproximadamente 0,3-1,0 μm de diâmetro situados na margem do eritrócito ou próximo a ela. Os sinais clínicos podem incluir icterícia sem hemoglobinemia e hemoglobinúria, febre, perda de peso, abortamento, letargia e, frequentemente, morte de animais com mais de dois anos de idade. É uma infecção endêmica em áreas tropicais e subtropicais, onde o principal vetor, o carrapato *Rhipicephalus microplus*, também é considerado endêmico. A criação consorciada de diferentes espécies é uma prática comum em fazendas de pequenos produtores no nordeste do Brasil, facilitando a transmissão de patógenos entre diferentes espécies de animais. Um estudo com búfalos relatou que todos estavam infectados por um isolado de *A. marginale* previamente detectada em bovinos. Os animais foram criados em uma fazenda de co-pastejo. Estudos moleculares da infecção por *A. marginale* em pequenos ruminantes são escassos. Existe apenas um relato sobre a detecção molecular de *A. marginale* em pequenos ruminantes no Brasil. Nesse sentido, objetivou-se estudar as interações entre *A. marginale*, caprinos, outros animais e possíveis vetores, elucidando a transmissão e manutenção do patógeno. Um total de 195 amostras de sangue de 119 caprinos, 71 ovinos e 5 bovinos. Foram coletadas e rastreadas para *A. marginale* usando primers visando o gene *msp4* por PCR. Dois dos 119 (1,68%, IC 95%: 0,46% - 5,92%) e 47/119 (39,5%, IC 95%: 31,17% - 48,48%) caprinos foram positivos para *Anaplasma* sp. por PCR e ELISA, respectivamente. Apenas 2/71 (2,82%, IC 95%: 0,78% - 9,7%) ovinos foram soropositivos para *Anaplasma* sp. por ELISA e todos foram negativos por PCR. Todos os bovinos testados foram negativos para *Anaplasma* sp. por PCR e ELISA. O sequenciamento da região microssatélite da MSP1a revelou que os animais foram infectados pelo genótipo B de *A.* marginale, além de terem sido encontradas as tandem repeats F e 91. Nossos resultados destacaram que os caprinos podem atuar como fonte de infecção de *A. marginale* para os bovinos ou ainda, como reservatório.

Palavras chave: Anaplasmose bovina. Co-pastejo. MSP. PCR. Pequenos ruminantes.

BARBOSA, Iago Carvalho. ***Anaplasma marginale* in a Multi-Species Grazing Farm in the State of Paraiba, Northeastern Brazil.** 2019. 50 p. Dissertation (Master's Degree in Animal Science) – State University of Londrina, Londrina, 2019.

ABSTRACT

Anaplasma marginale is the etiological agent of bovine anaplasmosis. It belongs to the genus *Anaplasma* and to the family Anaplasmatacae of the order Rickettsiales which appear as dense, rounded, intraerythrocytic bodies, approximately 0.3–1.0 µm in diameter situated on or near the margin of the erythrocyte. Clinical signs may include icterus without hemoglobinemia and hemoglobinuria, fever, weight loss, abortion, lethargy, and often death in animals over two years old. It is an endemic infection in subtropical and tropical areas, where the main transmissible agent, the tick *Rhipicephalus microplus*, is also considered endemic. Multispecies grazing is a common practice on smallholder farms in the northeast of Brazil, facilitating the transmission of pathogens within different herd species. A study with buffaloes, reported that they were infected by an *A. marginale* strain detected previously in cattle. The animals were breed in a co-grazed farm. Molecular studies of *A. marginale* infection in small ruminants are scarce. There is only one report regarding the molecular detection of *A. marginale* in small ruminants in Brazil. Accordingly, the aim was to study the interactions of *A. marginale* with goats, other co-grazed animals, and possible vectors, elucidating the transmission and maintenance of the pathogen A total of 195 blood samples from 119 goats, 71 sheep, and 5 cattle were collected and screened for *A. marginale* using primers targeting the *msp4* gene by PCR. Two out of 119 (1.68%, 95% CI: 0.46% – 5.92%) and 47/119 (39.5%, 95% CI: 31.17% – 48.48%) goats were positive for *Anaplasma* sp. by PCR and ELISA, respectively. Only 2/71 (2.81%, 95% CI: 0.78% – 9.7%) sheep were seropositive for *Anaplasma* sp. by ELISA and all were negative by PCR. All cattle tested negative for *Anaplasma* sp. by PCR and ELISA. Sequencing of the microsatellite region of MSP1a revealed that animals were infected by *A. marginale* genotype B and repeats F and 91. Our results highlighted that goats may act as *A. marginale* source of infection to cattle or as principal reservoir host.

Key words: PCR. Bovine anaplasmosis. Small ruminants. MSP. Co-grazing**.**

LISTA DE ABREVIATURAS E SIGLAS

CAT	Card agglutination test
CF	Complement fixation
cPCR	Conventional Polymerase Chain Reaction
cELISA	Competitive Enzyme-linked Immunosorbent Assay
ELISA	Enzyme-linked Immunosorbent Assay
HVRs	Central Hypervariable Regions
IFAT	Indirect Fluorescent Antibody Test
IgG	Immunoglobulin G
Mab	Monoclonal Antibody
MSP	Major Surface Protein
nPCR	Nested Polymerase Chain Reaction
PCR	Polymerase Chain Reaction
PCV	Packed Cell Value
qPCR	Quantitative Polymerase Chain Reaction
UTRs	Untranslated Regions

SUMARY

1. INTRODUCTION

Anaplasma marginale is the etiological agent of bovine anaplasmosis. It belongs to the genus *Anaplasma* in the Anaplasmataceae family, and the order Rickettsiales. It appears as dense, rounded, intraerythrocytic bodies, approximately 0.3–1.0 µm in diameter situated on or near the margin of the erythrocyte (OIE - WORLD ORGANIZATION FOR ANIMAL HEALTH, 2015). The agent was first described by Arnold Theiler in 1910, who reported "marginal points" in stained erythrocytes of sick cattle in Africa (THEILER, 1910). Clinical signs may include icterus without hemoglobinemia and hemoglobinuria (KOCAN et al., 2010a), fever, weight loss, abortion, lethargy, and often death in animals over two years old (KOCAN et al., 2003).

The disease is considered endemic in tropical and subtropical areas (~40^{o}N-32^{o}S), but it is also found in temperate zones (AUBRY; GEALE, 2011; KOCAN et al., 2010a). The bovine anaplasmosis is also seen in other ruminants, including water buffalo (*Bubalus bubalis*), bison (*Bison*), African antelopes, and some species of deer can become infected (AUBRY; GEALE, 2011).

Anaplasma marginale is transmitted primarily by ticks. Many species of ticks were reported as capable of transmitting the organism, but the most common vectors are *Dermacentor* spp. (*D. andersoni*, *D. variabilis* and *D. albipictus*) and *Rhipicephalus* spp. (*R. microplus* and *R. annulatus*) (KOCAN et al., 2004). However, mechanical transmission can be achieved by any means of transferring infected erythrocytes, such as blood contaminated mouthparts of biting flies and by instruments frequently used in veterinary practice (KOCAN et al., 2003, 2010b).

Anaplasma infection is frequently diagnosed by serological and molecular methods. Serological methods are frequently used to test large herds and chronic cases (COETZEE et al., 2007; STUEN; GRANQUIST; SILAGHI, 2013). However, the analysis of the genome and molecular characterization of major surface proteins (MSPs) are more specific and reliable than other techniques (KOCAN et al., 2003). The direct visualization under the microscope of stained blood smears is not a reliable technique, once it has low sensibility (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015).

Multispecies grazing is a common practice on smallholder farms in Northeastern Brazil, which may facilitates the transmission of pathogens within different herd species (DA SILVA et al., 2018). The contact between different species may leads to the dissemination of pathogens through vectors or indirect contact. A previous study has showed

that the same strain of *A. marginale* was capable to infect buffaloes co-grazed with cattle, ticks found on the buffaloes and cattle in areas nearby (SILVA et al., 2014b).

In small ruminants, only two studies have been able to detect *A. marginale*. In Iran, two out of 200 sheep were detected positive for the agent using a set of primers targeting the *msp4* gene of the bacterium by conventional PCR (YOUSEFI, 2018). A previous study conducted by our research group has detected 11/403 (2.73%) goats PCR-positive for *A. marginale msp4* gene in the State of Paraiba, northeastern Brazil (DA SILVA et al., 2018).

In order to elucidate the epidemiology of *A. marginale* in the farm from the state of Paraíba (DA SILVA et al., 2018), this study was conducted.

2. BOVINE ANAPLASMOSIS AND *A. marginale* – REVIEW

2.1. INTRODUCTION

Anaplasmosis refers to a disease of ruminants caused by obligate intra-erythrocytic bacteria of the genus *Anaplasma*. The disease is more common in cattle, but other ruminants can also become persistently infected (AUBRY; GEALE, 2011). It is a non-contagious disease, that is spread mainly by a biological vector, the tick. However, mechanical vectors such as biting flies or blood-contaminated fomites, like surgical equipment, can also transmit the agent (AUBRY; GEALE, 2011).

It is considered an endemic disease in tropical and subtropical areas (~40^{o}N-32^{o}S), including South and Central America countries, the United States (US), the south of Europe, Africa, Asia, and Australia, but also found in temperate zones (AUBRY; GEALE, 2011; KOCAN et al., 2010a). In the countries of Mexico, Central and South America and the Caribbean, the bovine anaplasmosis is considered endemic (KOCAN et al., 2003). The bovine anaplasmosis is also seen in other ruminants including water buffalo, bison, African antelopes, and some species of deer can become infected (AUBRY; GEALE, 2011).

2.2. BOVINE ANAPLASMOSIS

The etiological agent of bovine anaplasmosis is *Anaplasma marginale*. The bacterium belongs to the genus *Anaplasma* and to the family Anaplasmataceae of the order Rickettsiales. *A. marginale* organisms appear as dense, rounded, intraerythrocytic bodies approximately 0.3–1.0 µm in diameter situated on or near the margin of the erythrocyte (OIE - WORLD ORGANIZATION FOR ANIMAL HEALTH, 2015). The agent was first described by Arnold Theiler in 1910, which reported “marginal points” in stained erythrocytes of sick cattle in Africa (THEILER, 1910).

Anaplasma marginale infection results in considerable economic losses to both beef and dairy industries worldwide (KOCAN et al., 2010a). The agent infects erythrocytes that are phagocytized by the reticuloendothelial host system, resulting in mild-to-severe hemolytic anemia and icterus without hemoglobinemia and hemoglobinuria (KOCAN et al., 2010a). Clinical signs may also include fever, weight loss, abortion, lethargy, and often death in animals over two years old (KOCAN et al., 2003). In vertebrate hosts, *A. marginale* incubation period may vary from 7-60 days (KOCAN et al., 2010a). During acute infection, ≥ 70% of erythrocytes may be parasitized and bacteremia may exceed 10^9 organisms per mL

(PALMER; BROWN; RURANGIRWA, 2000). Cattle that survive to acute infections become persistently infected animals, with a cyclic, low-level rickettsemia (10^2-10^7 organisms per mL of blood) (KOCAN et al., 2003, 2012; PALMER; BROWN; RURANGIRWA, 2000). These persistent infected animals develop life-long immunity and are resistant to the clinical disease (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015).

2.2.1. Transmission

The bovine anaplasmosis is a vector-borne disease, transmitted primarily by a biological vector, the tick. However, mechanical transmission can be achieved by any means of transferring infective erythrocytes, such as blood contaminated mouthparts of biting flies and by instruments frequently used in veterinary practice (KOCAN et al., 2003, 2010b). In places where tick vectors have proven to be absent or incapable to transmit the strain, the mechanical transmission may be the only way transmitting the *A. marginale* (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). The mechanical transmission is many times underappreciated as a mean of spreading the disease, but persistently infected cattle are a source of infection for both biological and mechanical transmission. Indeed, the biological transmission has been shown to be more efficient than mechanical transmission (KOCAN et al., 2010a).

Many species of ticks were reported as capable of transmitting the bacterium, but the most common vectors are *Dermacentor* spp. (*D. andersoni*, *D. variabilis* and *D. albipictus*) and *Rhipicephalus* spp. (*R. microplus* and *R. annulatus*) (KOCAN et al., 2004). After the blood-meal *A. marginale* infects the tick midgut cells, where non-infective reticulated forms replicate in large membrane-bound vacuoles or colonies, and became infective, as dense forms. Then, *A. marginale* invades other tissues as the salivary glands and may be efficiently transmitted (BRAYTON, 2012).

Transplacental transmission occurs in cattle resulting in healthy but persistently infected calves, showing that transplacental transmission may be important in the maintenance of *A. marginale*-infected herds (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). Previous studies have shown 5.9% to 41% of *A. marginale*-positive calves born from chronically infected dams (COSTA et al., 2016; DA SILVA; ANDRÉ; MACHADO, 2016; GRAU et al., 2013; SILVA; CASTRO; FONSECA, 2014; SILVA et al., 2015c; SILVESTRE et al., 2016).

2.2.2. Diagnosis

Anaplasma spp. infection is frequently diagnosed by serological and molecular methods. Serological methods are used frequently to test large herds and chronic cases (COETZEE et al., 2007; STUEN; GRANQUIST; SILAGHI, 2013). However, the analysis of the genome and molecular characterization of MSPs are more specific and reliable than other techniques. Information as geographical location, season, clinical signs and necropsy findings can be helpful in the bovine anaplasmosis diagnosis (KOCAN et al., 2003). The direct visualization under the microscope of stained blood smears is a not reliable technique in animals when parasitemia level is low and in persistent infected animals, where the inclusion bodies can be easily confused (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). In the acute phase of the disease blood smears may be used, when detecting levels are higher than 10^6 infected erythrocytes per mL (AUBRY; GEALE, 2011; TORIONI DE ECHAIDE et al., 1998).

A wide variety of serological assays were developed, such as complement fixation (CF) test, capillary-tube agglutination test, and indirect fluorescent antibody test (IFAT) (GONZALEZ; LONG; TODOROVIC, 1978; MOLLOY et al., 1999). Serologic tests before the development of enzyme-linked immunosorbent assay (cELISA) using MSP5, lack in sensitivity (BRADWAY et al., 2001; BRAYTON et al., 2005; CHUNG et al., 2014). Despite serological technique based on MSP5 surface-exposed protein showed high sensitivity and specificity, subsequently was found cross-reaction with other agents from the genus *Anaplasma* due to the conservation of the *msp5* gene (KOCAN et al., 2012; KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). Card agglutination test (CAT) can be performed, which is a sensitive and non-time-consuming technique that can also be conducted in the field. However, like other serological, the method can present nonspecific reactions (OIE - WORLD ORGANIZATION FOR ANIMAL HEALTH, 2015).

The molecular methods of diagnosis, such as PCR, allow the determination of the species based on the genetic material analysis, but it is non-practical technique for large scale surveillance (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). Despite the development of PCR techniques that can be done in large scale (CHAISI et al., 2017; HOVE et al., 2018a, 2018b; KHUMALO et al., 2016), the technique is economic impractical due to the high cost. Finally, PCR should be used as a definitive diagnostic tool for the infection.

2.2.3. Molecular Interaction And Major Surface Proteins

Ligands and receptors involved in host-pathogen interaction are of great importance in the development of control strategies. The MSPs of *A. marginale* are relevant, once they are considered to be adhesins (DE LA FUENTE et al., 2010). The MSP1a are adhesins to bovine erythrocytes (DE LA FUENTE et al., 2001a), tick cell extracts (DE LA FUENTE et al., 2001a) and the gut cells of ticks (DE LA FUENTE et al., 2001b). The binding domains of MSP1a are located in the tandem repeat region of this protein (DE LA FUENTE et al., 2003). Erythrocytes receptors are believed to be comprised of both protein and carbohydrate (DE LA FUENTE et al., 2010).

The cattle are the specific host for *A. marginale*. However, molecular studies have demonstrated a worldwide diversity of strains according to the major surface proteins (MSPs) and other genes (AWAD et al., 2011; DE LA FUENTE et al., 2010; MTSHALI et al., 2007; RUYBAL et al., 2009).

The genetic diversity of *A. marginale* has been classified using MSPs, such as MSP1a, MSP4, and MSP5, encoded by single genes (AUBRY; GEALE, 2011) and the proteins MSP1b, MSP2 e MSP3, encoded by multi-gene families (BRAYTON et al., 2005; OBERLE; PALMER; BARBET, 1993; PALMER et al., 1994; VISSER et al., 1992). The gene *msp1α*, which encodes MSP1a and MSP1b, shows a wide genetic diversity and has been used for the identification of *A. marginale* strains. It is a stable marker of strain identity during acute and persistent rickettsemia in cattle and ticks, but it does not provide information about the geographical origin (CABEZAS-CRUZ et al., 2013; DE LA FUENTE et al., 2007; KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015; PALMER; RURANGIRWA; MCELWAIN, 2001). The *msp4* gene is used as a stable marker providing strain identity and phylogeographic information (DE LA FUENTE et al., 2010; DE LA FUENTE; VAN DEN BUSSCHE; KOCAN, 2001). The *msp5* gene is extremely conserved between isolates of *A. marginale*, but rather used in the molecular diagnosis of infection by this rickettsia and its product, MSP5, is used for serological diagnosis of the disease (TORIONI DE ECHAIDE et al., 1998).

MSP1a and MSP1b are part of the MSP1 complex, composed of two structurally unrelated polypeptides (MACMILLAN et al., 2006). These proteins are involved in the adhesion of *A. marginale* to bovine erythrocytes, while only MSP1a is involved in the adhesion of the agent to tick cells (GARCIA-GARCIA et al., 2004). Many strains were found based on the MSP1a protein sequence. This protein is composed by a C-terminal conserved

domain and an N-terminal variable domain composed of one or more peptides of 23 to 31 amino acids similar among them and known as repeats (BARIGYE et al., 2004; LOPEZ et al., 2007; RIDING et al., 2003). MSP1a is used as a molecular marker and provides phylogenetic and evolutionary information about *A. marginale* strains (PALMER et al., 2004). It evolves under the positive selective pressure of the host immune system and molecular weight difference of the peptides in geographic strains is the result of variations in the numbers of tandem repeat units (PALMER et al., 2004).

The MSP2 and MSP3 are highly conserved immunodominant proteins, which start antigenic variation during rickettsemic peaks to evade the host existing immune response, maintaining the infection (BRAYTON et al., 2005, 2003). *A. marginale* uses recombinant mechanisms to generate antigenic variation in the MSP2 and MSP3 proteins, using all or part of the central hypervariable regions (HVRs) of donor pseudogenes (BRAYTON et al., 2005, 2003).

Information available on genetic diversity of *A. marginale* found an extensive diversity of *A. marginale* strains result of natural adaptation processes and pressure exerted on *A. marginale*, mainly in areas where there is an intense cattle movement (QUIROZ-CASTAÑEDA; AMARO-ESTRADA; RODRÍGUEZ-CAMARILLO, 2016). This diversity complicates control strategies and treatments since the strains can have different behaviors and characteristics, becoming hard to develop a cross-protective vaccine or antimicrobials (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). Previous studies have shown that the infection-exclusion occurs in cattle and ticks. In an infection-exclusion establishment, one strain of *A. marginale* prevents that a second strain infects the animal after a challenge exposure (BASTOS et al., 2010; DE LA FUENTE et al., 2002; DE LA FUENTE; BLOUIN; KOCAN, 2003). However, previous studies also shown that superinfection can happen (PALMER; BRAYTON, 2013; UETI et al., 2012). Superinfection is characterized by the infection of different strains, found in the same environment, infecting the same animal (PALMER et al., 2004).

2.2.4. Prevalence In Brazil

The incidence of bovine anaplasmosis is associated to climatic and management factors that can contribute to the presence of vectors in the environment, as happen in tropical and subtropical areas (COSTA et al., 2013). The Brazilian territory has a continental dimension and the climatic characteristics vary greatly (GONÇALVES, 2000). The relation between bovine anaplasmosis and its vector population generate an

epidemiological picture. In the first screen, the vector population have a variation that can be due to climatic conditions or implementation of wrong control strategies (ARAÚJO et al., 2003). When cattle, not exposed for a long period, have contact with the agent, those animals manifest the disease which can leads high mortality rates, classified as a typical case of enzootic instability (DE OLIVEIRA; PEDREIRA; ALMEIDA, 1992; RIBEIRO et al., 1984). In the second screen, the vector is present all the time. In these areas, the cattle have a great resistance to the infection, developing immunity still young when the colostral antibodies protect against the infection of *A. marginale*, and become persistent infected animals (ARAÚJO et al., 2003). In the enzootic stable areas, outbreaks or high abortion rates are not expected (MELO et al., 2001). Finally, in the third screen, the climatic conditions are not favorable to the maintenance and development of vector, nevertheless it enables temporary infection in population at risk (ARAÚJO et al., 2003).

The *A. marginale* epidemiological situation in most of the regions in Brazil is of enzootic stability (ARAÚJO et al., 2003). In a previous study conducted in Australia, was observed that when 75% of the calves under 9 months were exposed to ticks *R. microplus* infected with *B. bovis*, clinical manifestation of babesiosis were not seen in the herd (MAHONEY; ROSS, 1972). This concept was further extrapolated for all tickborne diseases worldwide. However, the epidemiology of the prevalence of *A. marginale*, the occurrence of clinical manifestation and the mortality rates due to the infection are far more complex than what was seen in the *R. microplus* - *B. bovis* relation (JONSSON et al., 2012).

Due to the country size, some states in Brazil can be enzootically stable Acre (BRITO et al., 2002), Rondônia (BRITO et al., 2010), Piauí (SOUZA et al., 2013), Bahia (ARAÚJO et al., 1998; BARROS et al., 2005; COSTA et al., 2016) and Goiás (SANTOS; LINHARES; MADRUGA, 2001), or enzootically unstable, such as Sergipe (DE OLIVEIRA; PEDREIRA; ALMEIDA, 1992), Pernambuco (SANTOS et al., 2017), Mato Grosso do Sul (SILVA et al., 2015a) and Santa Catarina (CANEVER et al., 2014). There are also states that are considered as enzootically stable and unstable areas, like Pará (SILVA et al., 2014a, 2015a), Tocantins (SILVA et al., 2015a; TRINDADE; ALMEIDA; FREITAS, 2011), Paraíba (COSTA et al., 2013, 2011; COSTA; SIMÕES; RIET-CORREA, 2009; SOUZA et al., 2001), Minas Gerais (BARBIERI et al., 2016; CARVALHO et al., 2012; MELO et al., 2001; PEREIRA; GUIMARÃES; ROCHA, 2009), Mato Grosso do Sul (MADRUGA; AYCARDI; PUTT, 1983; SILVA et al., 2015a), Rio de Janeiro (SILVA et al., 2014b, 2015c) and Paraná (ANDRADE et al., 2001; MARANA et al., 2009; SOTT et al., 2016; VIDOTTO et al., 1998; YOSHIHARA et al., 2003).In the north of Brazil, the Pará state

showed a seroprevalence of 68.3% (112/164) and 74.5% (506/ 679) by indirect ELISA (iELISA) (GUEDES JUNIOR et al., 2008; SILVA et al., 2014a) and in the Tocantins state two prevalence were found by iELISA, 52.6% and 89.9%, presenting areas of enzootic stability and instability in the same state (SILVA et al., 2015a; TRINDADE et al., 2011). The molecular prevalence in all the north region ranges from 92.4% to 98.6% by PCR for the *msp5* gene (BRITO et al., 2010).

In the northeast region of Brazil, the Bahia state showed seroprevalence of 97.2% (105/108) by ELISA, 97.2% (105/108) by IFAT and 90.7% (98/108) by CAT (ARAÚJO et al., 1998); by ELISA was also found a seroprevalence 97.8% (BARROS et al., 2005), 69.1% (65/94) (COSTA et al., 2016) and 96.9% (314/324) (MADRUGA et al., 2000); and, by IFAT was reported a seroprevalence of 97.2% (315/324) (MADRUGA et al., 2000). Previous studies conducted in Ibicaraí, Bahia, showed a prevalence of 31.1% (96/309) by blood smear and 63.1% (195/ 309) by nested PCR (nPCR) of the *msp5* gene on the same samples (AMORIM et al., 2014); and, a prevalence of 69.1% (65/94) by iELISA and 30.8% (37/120) by nPCR of the msp1α gene on the same samples. The prevalence of the techniques differs because the molecular methods present higher sensibility in comparison to direct blood smear analysis (AUBRY; GEALE, 2011). In Paraíba state, the seroprevalence of 16.5% (42/255) by IFAT was found (COSTA et al., 2013). Also, outbreaks of the Sertão region of Paraíba state showed a low prevalence of 8.5% (13/153) by blood smears with a high parasitemia of 1.8-80% (COSTA et al., 2011), which present the low sensitivity of technique (AUBRY; GEALE, 2011). Seroprevalences of 67.3% (290/431) in Pernambuco (SANTOS et al., 2017), 89.1% (180/202) in Piauí (SOUZA et al., 2013) and 12.3% (319/2593) in Sergipe (DE OLIVEIRA; PEDREIRA; ALMEIDA, 1992) by IFAT were found.

In the Midwest region, the state of Goiás found a seroprevalence of 99.2% (517/521) and 96.9 (505/521) by ELISA and IFAT, respectively (SANTOS; LINHARES; MADRUGA, 2001). Other study conducted in Goiás comparing four different diagnostics assays, blood smear, IFAT, ELISA and quantitative PCR (qPCR), showed a prevalence of 34% (17/50), 50% (25/50), 54% (27/50) and 38% (19/50), respectively (MACHADO et al., 2015). Also, when comparing calves, heifers, pregnant and lactating cows with each other, the calves and heifers were more likely to die from anaplasmosis than lactating cows. Additionally, the calves were taken from free areas to infested tick areas prior to the outbreak and a high number of flies were reported in the property (MACHADO et al., 2015). In Mato Grosso state was reported a prevalence of 41.1% (215/516) by iELISA (SILVA et al., 2015a), while in Mato Grosso do Sul state, a seroprevalence of 100% (50/50) by CAT (MADRUGA

et al., 1985), 7.95% (53/667) by CF (MADRUGA; AYCARDI; PUTT, 1983) and 43.9% (56/128) by ELISA (SILVA et al., 2006).

In the southeast region, the state of Minas Gerais has a prevalence that ranges from 92% (81/88) to 96.2% (385/400) (BARBIERI et al., 2016; CARVALHO et al., 2012; MELO et al., 2001; PEREIRA; GUIMARÃES; ROCHA, 2009). Previous studies, conducted in the mesoregion of Zona da Mata, showed the prevalence of 35% (14/40) by blood smear (PAULA et al., 2015) and 81.1% (257/317) and 73.5% prevalence (233/317) by IFAT and CAT, respectively. In other study was found a prevalence of 48% (48/100) by blood smears, with bacteremia ranging from 0.1 to 16.1%, and 70.2% (66/94) of positivity by qPCR on the same samples (POHL et al., 2013). In the state of Rio de Janeiro, seroprevalences range from 31.4% (13/41) to 98,21% (219/223) by iELISA (SILVA et al., 2015b; SOUZA et al., 2001). Studies showed that cows that were pregnant or lactating, mainly lactating for the first time, presented high odds ($p<0.05$) to be seropositive. In contrast, cows seroprevalence decreased during the peripartum (SILVA; FONSECA, 2013). Also, other studies conducted in Rio de Janeiro showed that tick infestation, breed, production type (milk/beef), age and stock density are related to the infection of *A. marginale* (SILVA; CASTRO; FONSECA, 2014; SILVA et al., 2015b). In the state of Sao Paulo, the seroprevalence by ELISA and IFAT was of 80% (16/20) (DA SILVA; ANDRÉ; MACHADO, 2016). A study conducted during an outbreak in the municipality of Lins showed low seroprevalence (58% (29/50) by ELISA and 52% (26/50) by IFAT) (MACHADO et al., 2015), probably associated to the low levels of antibodies during the acute phase (AUBRY; GEALE, 2011). In the same study, was found higher prevalence by blood smear and PCR of *mspα* gene, 84% (42/50) and 94% (47/50) respectively (MACHADO et al., 2015), showing that molecular and direct techniques are more reliable with high levels of rickettsemia (PALMER; BROWN; RURANGIRWA, 2000). Another study corroborates with the previous findings, which the prevalence of 100% (20/20) positivity based on the msp1α gene analysis was found (DA SILVA; ANDRÉ; MACHADO, 2016). Conflicting to the data, records from the Veterinary Hospital at the São Paulo University, in Botucatu, showed a low prevalence of 31.5% (361/1147) by blood smear analysis (GONÇALVES et al., 2011). The study also found higher mortality rates in calves between 2-6 months (24.7% (283/1147), the disease increased during the fall and European cattle (*Bos Taurus*) and crossbreed cattle showed higher infection rates (GONÇALVES et al., 2011). In the state of Espirito Santo was found a prevalence of 17.6% (32/182) by blood smear analysis (JÚNIOR et al., 2008).

In the South region of Brazil, the Paraná state showed different seroprevalence of 92,9% (658/708) (ANDRADE et al., 2001), 58.7% (131/223) (MARANA et al., 2009), 87,56% (359/410) (VIDOTTO et al., 1998) and 76,1% (172/226) (YOSHIHARA et al., 2003) by cELISA; 71.1% (64/90) (ELIAS, 2016) by iELISA; 77.7% (267/344) (SOTT et al., 2016) by ELISA; and 67.4% (281/417) (VIDOTTO et al., 1997) by IFAT. In the state of Rio Grande do Sul, a seroprevalence of 64% (794/1246) by CAT was found (ARTILES, 1995) and in the state of Santa Catarina was reported as 86% (516/600) by IFAT (DALAGNOL; MARTINS; MADRUGA, 1995). By multiplex PCR of the *msp1α* gene, was found a prevalence of 60.6% (20/33) during an outbreak in a beef herd (CANEVER et al., 2014). Despite age did not seem to be a factor in the outbreak, the presence of known vector ticks (*R. microplus*) was reported and may be related to the infection (CANEVER et al., 2014).

2.2.5. Control Measures And Chemotherapy

In endemic regions, cattle develop resistance to ticks and bovine anaplasmosis (JONGEJAN; UILENBERG, 2005). Control measures include the use of acaricides, chemotherapy, chemoprophylaxis, controlled exposure, and vaccination. Despite all, the measures only seem to limit the losses (KOCAN; DE LA FUENTE; BLOUIN, 2008). Acaricides are selecting resistant populations of ticks and leaving residues in the meat and milk, concerning the public health (THULLNER; WILLADSEN; KEMP, 2007). Chemoprophylaxis and controlled exposure to infected ticks may or may not be effective to develop immunity in the animals. Therefore, immunoprophylaxis seems to be the better choice to control the disease (KUTTLER, 1984; VIZCAINO et al., 1980). Despite all the complete genome sequences known, develop of vaccines that can induce protection against an array of strains of *A. marginale* is still pending (QUIROZ-CASTAÑEDA; AMARO-ESTRADA; RODRÍGUEZ-CAMARILLO, 2016).

The isolation and identification of *A. marginale* provide the information of how this rickettsia is distributed all over the world (QUIROZ-CASTAÑEDA; AMARO-ESTRADA; RODRÍGUEZ-CAMARILLO, 2016). Vaccination is proposed as the most effective tool for the prevention of infectious diseases. With the continuous sequencing of genomes, the availability of the information has led to a new paradigm vaccine development (BAMBINI; RAPPUOLI, 2009; QUIROZ-CASTAÑEDA; AMARO-ESTRADA; RODRÍGUEZ-CAMARILLO, 2016).

2.2.6. *Anaplasma marginale* in Small Ruminants

Multispecies grazing is a common practice on smallholder farms in the northeast of Brazil, facilitating the transmission of pathogens within the different herd species (DA SILVA et al., 2018). A study of co-grazing ruminants has shown a single *A. marginale* strain infecting buffaloes coexisting with cattle, ticks found on the buffaloes, and cattle breed neraby (SILVA et al., 2014b).

In small ruminants, only two studies were able to detect *A. marginale*. In Iran, two out of 200 sheep were detected positive for the agent by PCR of the *msp4* gene (YOUSEFI, 2018). Another study conducted in Brazil found a prevalence in goats of 2.73% (11/403) by PCR of the *msp4* gene (DA SILVA et al., 2018). The samples were collected in the northeast of Brazil (state of Paraíba), which multispecies grazing of cattle, sheep, and goats was observed. Also, the presence of ticks and flies were confirmed which may influence in the spread of pathogenically agents within the different herd species (DA SILVA et al., 2018).

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3. OBJECTIVES

3.1. GERAL OBJECTIVE

- To detect and identify the *A. marginale* genotype in goats, sheep and cattle from a multispecies grazing system, Paraíba State, northeastern Brazil.

3.2. SPECIFIC OBJECTIVES

- To screen goat, sheep and cattle blood samples for the presence of *A. marginale* infection by PCR assays targeting the *msp4* gene;
- To screen goat, sheep and cattle blood samples for the presence of *Anaplasma* spp. infection by serological assays targeting the MSP5 protein;
- To molecular characterize *A. marginale msp1α* and *msp4* genes;
- To identify the *A. marginale* genotype and strain;
- Sampling and identification of ticks feeding on animals.

4. ARTICLE

ANAPLASMA MARGINALE IN A MULTI-SPECIES GRAZING FARM IN THE STATE OF PARAIBA, NORTHEASTERN BRAZIL

ANAPLASMA MARGINALE EM UMA FAZENDA DE CRIAÇÃO CONSORCIADA NO ESTADO DA PARAÍBA, NORDESTE DO BRASIL

ABSTRACT

Anaplasma marginale is the etiological agent of bovine anaplasmosis, which causes significant economic losses for cattle industries and is increasingly being detected in other animal species. Many species of ticks were reported as capable of transmitting the organism, with *Dermacentor* spp. and *Rhipicephalus* spp. ticks the most common vectors. To date, only two studies have detected *A. marginale* in small ruminants, although the role of small ruminants in the epidemiology of the pathogen remains unknown. Accordingly, this study aimed to detect and genotype *A. marginale* in goats, sheep and cattle from a multispecies grazing system in Paraíba State, northeastern Brazil. For this purpose, blood samples from goats (n= 119), sheep (n = 71), and cattle (n = 5) were collected in July 2018. All samples were screened by PCR targeting the *Anaplasma* spp. *msp4* gene and by an indirect ELISA assay using MSP5 protein as antigen. Additionally, *msp4* PCR-positive samples were submitted to a PCR targeting the *msp1a* gene in order to genotyping the *A. marginale* genotype. Two out of 119 (1.68%, 95% CI: 0.46% – 5.92%) and 47/119 (39.5%, 95% CI: 31.17% – 48.48%) goats were positive for *Anaplasma* sp. by PCR and ELISA, respectively. Only 2/71 (2.81%, 95% CI: 0.78% – 9.7%) sheep were seropositive for *Anaplasma* sp. by ELISA and all were negative by PCR. All cattle tested negative for *Anaplasma* sp. by PCR and ELISA. Sequencing of the microsatellite region of MSP1a revealed that animals were infected by *A. marginale* genotype B and repeats F and 91. Our results highlighted that goats may act as *A. marginale* source of infection to cattle.

Key words: Bovine anaplasmosis, small ruminants, goats, sheep, *msp1α* and *msp4*.

Introduction

Anaplasma marginale is an obligate intra-erythrocyte bacterium, known to cause bovine anaplasmosis (AUBRY; GEALE, 2011). This bacterium is endemic in tropical and subtropical areas (AUBRY; GEALE, 2011; KOCAN et al., 2010a), and causes great economic losses in the bovine and milk industry worldwide (KOCAN et al., 2010a). Clinical signs of the disease may include icterus without hemoglobinemia and hemoglobinuria (KOCAN et al., 2010a), fever, weight loss, abortion, lethargy, and often death in animals over two years old (KOCAN et al., 2003). The animals that survive acute disease may develop persistent infection and become reservoirs of *A. marginale* (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). Antigenic variations of *A. marginale* outer membrane proteins (OMPs) allow evading the host's immune system and maintaining a persistent infection (ALLEMAN et al., 1993; BRAYTON et al., 2001; PALMER et al., 1994).

Many species of ticks are implicated in the transmission of *A. marginale*, but the most common vectors belong to the genus *Dermacentor* and *Rhipicephalus* (KOCAN et al., 2004). In Brazil, bovine anaplasmosis is biologically transmitted by *Rhipicephalus microplus* ticks (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015; MA et al., 2016), which may also found feeding on small ruminants (BRITO; SANTOS; GUERRA, 2005). A study conducted in Rio de Janeiro, showed that buffaloes co-grazed with cattle infested with *R. microplus* ticks were infected by the same *A. marginale* strain detected in cattle (SILVA et al., 2014). However, mechanical transmission can be achieved by any means of transferring infective erythrocytes, such as blood contaminated mouthparts of biting flies and by instruments frequently used in veterinary practice (KOCAN et al., 2003, 2010b).

Anaplasma marginale has been described in different animal species, but studies involving the detection of this bacterium in small ruminants are scarce. Molecular detection of *A. marginale* in small ruminants was primarily described in sheep from Iran (YOUSEFI et al., 2017), and recently in goats (*Capra hircus*) from northeastern Brazil (DA SILVA et al., 2018). In the northeastern Brazil, multispecies grazing is a common practice among smallholder farms, facilitating interspecies transmission of pathogens (COSTA; SIMÕES; RIET-CORREA, 2009). The contact between different animal species may leads to the dissemination of pathogens through direct or indirect contact, as vectors and fomites. Accordingly, the aim of this study was to detect and genotyping *A. marginale* in goats, sheep and cattle from a multispecies grazing system in Paraíba State, northeastern Brazil.

Material and methods

With the approval from the Ethics Committee for Animal Experimentation and Animal Welfare of the Universidade Federal da Paraíba (protocol 3305/14), a total of 195 samples from 119 goats, 71 sheep, and 5 cattle were collected. An epidemiological questionnaire was given to the farm owner addressing age, gender, and presence of ticks. The age of goats was stratified into groups of higher or equal to one year and less than one year.

Goat, sheep and cattle blood samples were collected by venipuncture of the jugular vein using commercial tubes containing EDTA and separating gel clots (BD Vacutainer®, Franklin Lakes, NJ, USA). After, the packed cell volume (PCV) and plasma protein concentration were measured by routine centrifugation and refractometry techniques, all samples were stored at -20 ºC. A PCV of < 0.22 L/L and a plasma protein concentration of > 8 g/dL were used as indicators of anemia and hyperproteinemia, respectively (WEISS; WARDROP; SCHALM, 2010).

Ticks were removed using a commercial hook (ÓTom/Tick Twister®, H3D Inc., Lavancia, France), and kept in absolute ethanol-labeled tubes for further classification according to morphological taxonomic keys (ARAGÃO; FONSECA, 1961; GUIMARÃES; BATTESTI; TUCCI, 2001; ONOFRIO et al., 2006; PINTO et al., 2017).

Genomic DNA was extracted from 200 µL of whole blood using a commercial kit (QIAGEN, QIAamp DNA blood Mini Kit (250)), according to the manufacturer's instructions. Negative controls using ultra-pure water were performed in parallel to monitor cross-contamination in each batch of 20 samples. The concentration of the samples were measured using nanodrop.

PCR amplification of the caprine, ovine and bovine glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) housekeeping gene was done to verify successful DNA extraction, as previously described (BIRKENHEUER; LEVY; BREITSCHWERDT, 2003).

Samples were screened for *A. marginale* DNA using previously described primers targeting the *Anaplasma* spp. *msp4* gene (~870 bp) (DE LA FUENTE et al., 2007). A positive blood sample for *A. marginale* and ultrapure water were used as positive and negative controls, respectively. The amplified products were subjected to gel electrophoresis in 1.5% agarose gels for 50 minutes at 100 V, followed by SYBR safe staining (6 µg/mL; SYBR® Safe DNA Gel Stain, Invitrogen, CA, USA), and were viewed under a 312 nm UV light transilluminator. The gels were subsequently photographed using Kodak DC290 (New York, USA).

Amplified DNA fragments of the *msp4* gene from *Anaplasma* spp. isolates were directly sequenced using the Sanger method (SANGER; NICKLEN; COULSON, 1977) and analyzed sequences compared by BLASTn with those present in the GenBank1 database.

The sequencing of the *A. marginale msp1α* microsatellite was performed to determine the genotype. This microsatellite is located at the 5'-untranslated region (UTR) of the *msp1α* gene between the putative Shine–Dalgarno (GTAGG) sequence and the translation initiation codon (ATG) (DE LA FUENTE; VAN DEN BUSSCHE; KOCAN, 2001). The microsatellite structure is GTAGG (**G/ATTT**)m (**GT**)n **T** ATG (ESTRADA-PEÑA et al., 2009). where microsatellite sequence is in bold letters. The SD-ATG distance was calculated according to the equation (4 × m) + (2 × n) + 1. The *msp1α* gene was also used to characterize the strain according to variation in the number and sequence of tandem repeats at the 5' end of gene (ALLRED et al., 1990; PALMER; RURANGIRWA; MCELWAIN, 2001). The MSP1a repeats are named alphanumerically, in order to distinguish sequence variants, leading to *msp1α* genotypes. The software RepeatAnalyzer was used to determine the repeats found in the sequence (CATANESE; BRAYTON; GEBREMEDHIN, 2016).

The serum samples were sent to the UNESP (Universidade Estadual Paulista) in order to detect IgG antibodies to *A. marginale* by a commercial Enzyme-linked immunosorbent assay (ELISA) (Imunodot Diagnostics, Jaboticabal, BR). The preparations and procedures used followed the directions of the instruction manual for the product. At the end of the procedure, the plates were read using an ELISA microplate reader (Multiskan GO UV/VIS spectrophotometer; Thermo Scientific) with a 405 nm filter. The results were obtained by calculating the cutoff index, optical density and sample coloration, as indicated in the instruction manual. Positive samples for *A. marginale* presented an intense yellow color and optical density higher or equal to the cutoff index. Negative samples showed no intense yellow color and optical density lower than the cutoff index.

A non-parametric Mann–Whitney test was used to compare the PCV and plasma protein concentration between *Anaplasma*-infected and non-infected goats.

Results

All the samples successfully amplified the *gapdh* gene. We found that 2 out of 119 goats (1.68 %) were positive for *A. marginale* by PCR. Furthermore, 47/119 (39.5%) goats and 2/71 (2.81%) sheep were ELISA-positive for *A. marginale*, including the two goat PCR-positive samples. Finally, no cattle presented positivity for *A. marginal* by means of PCR and ELISA and no sheep was positive by PCR.

From the two positive *Anaplasma* spp. *msp4* gene samples, one showed 100% identity to multiple *A. marginale msp4* gene sequence deposited in GenBank (KX989516, KX989512, CP006847, CP006846, JN564647, AF428086, AF428083, AY665999, AY665997), and the other sample did not generate sequence.

The mean PCV concentration for goats was 0.21 L/L. A total of 62/119 goats were anemic. The mean PCV for positive goats was 0.13 L/L and 0.21 L/L for PCR and ELISA, respectively. The mean PCV for *A. marginale* positive goats by PCR and ELISA presented no significant association when compared with *A. marginale* negative goats (p^{PCR}=0.07855 and p^{ELISA}=0.8746).

The mean plasma protein concentration for goats was 6.67 g/dL. A total of 6/119 goats presented hyperproteinemia. The mean plasma protein concentration for positive goats was 6.8 g/dL and 6.93 g/dL for PCR and ELISA, respectively. The mean plasma protein concentration for *A. marginale* positive goats by PCR and ELISA presented no significant association when compared with *A. marginale* negative goats (p^{PCR}= 0.5821 and p^{ELISA}=0.9896).

The mean PCV and plasma protein concentration for sheep were 33.66 L/L and 6.84 g/dL. Only two sheep were anemic and no hyperproteinemic sheep was found. The PCV and mean plasma protein concentration for ELISA-positive sheep was and 33 L/L and 7.2 g/dL, respectively. The PCV and mean plasma protein concentration for *A. marginale* positive sheep by ELISA presented no significant association when compared with *A. marginale* negative goats (p^{PCV}=0.7242 and p^{PPC}=0.7637).

The analysis of MSP1a microsatellite sequences resulted in the genotype B (ESTRADA-PEÑA et al., 2009). The microsatellite sequence produced a distance SD-ATG of 23 nucleotides. By the analysis of the *msp1α* gene the tandem repeats were identified and the strain F/91 was found (CATANESE; BRAYTON; GEBREMEDHIN, 2016; KHUMALO et al., 2016).

All the ticks collected were identified as *Rhipicephalus microplus* species. Flies were also seen at the property, but it was not possible to collect and identify them.

Discussion

In the current project the samples were analyzed by PCR and ELISA using the *msp4* and *msp1α* genes, and MSP5 antigen, respectively. Previous studies conducted in Paraíba with cattle showed a seroprevalence of 16.5% (84/509) by IFAT (COSTA et al., 2013) and a prevalence ranging from 1.8-80% by blood smears analysis in 22 outbreaks

(COSTA et al., 2011). Regarding small ruminants, a study for the detection of *Anaplasma* spp. in the northeast found anti-*Anaplasma* spp. antibodies based on recombinant MSP5 of *A. marginale* (RAMOS et al., 2008). Direct molecular detection of *A. marginale* in goats was only reported in Iran (YOUSEFI et al., 2017) and recently, by our group, in Paraíba state (DA SILVA et al., 2018).

Multi-species grazing is a common practice among small farms in northeastern Brazil. This behavior allows the families to produce a wide diversification of products toward commercialization (DA SILVA et al., 2018). In Rio de Janeiro, a study showed similarities in *A. marginale* strains of buffaloes and cattle, also the same strain was found in *R. microplus* ticks collected from the buffaloes (SILVA et al., 2014). The repeat F was found in goats from Paraiba state, but the genotype could not be defined (DA SILVA et al., 2018). The characterization of the genotype is important to provide phylogenetic information about the genetic variation among the isolates, phylogeographical information, if the gene is under positive or negative selection pressure, if the same strain can infect more than one animal species and which is acting as source of infection. In order to detect the genotype, PCR targeting the *msp1α* is the best option.

Two out of 119 goats were *msp4* PCR positive for *A. marginale*, and no PCR-positive were seen for sheep and cattle. By serological MSP5 protein assay, 47/119 goats were ELISA-positive, including the two PCR positive samples. An explanation for the low prevalence found by PCR when compared to ELISA can be the low infestation of ticks *R. microplus* seen during the collection. Once the competent vector infestation is low in the environment, the agent cannot be spread among the herd, lowering the parasitemia levels of *Anaplasma* spp., and being unable the detection of the agent by PCR. The tick *R. microplus* has been described as the main vector of *A. marginale* (KOCAN et al., 2010). However, contact with the agent stimulates the immunological system of the host to produce antibodies. These antibodies can be detected for life by serological diagnostic assays, so the ELISA positive animals found were chronic infected for *Anaplasma* spp. The ELISA technique used in the present study has great sensibility, but do not present a high species-specificity (KOCAN et al., 2012; KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015), forward it is not possible to determine the species of anaplasma by this assay.

During rainfall periods, the infestation of ticks is higher. The property is located in the municipality of Olives, which stays within the microregion of occidental

Curimataú, Paraíba. During the collection month (July), the rainfall index was low (17.5 nm) at this region ("Meteorologia – Chuvas – AESA"). Additionally, the farm owner reported that there was no rainfall in the farm over the past few months. The data corroborates to the free vector areas observed and the low prevalence of *A. marginale* by PCR.

The ELISA-positive sheep found, could be due to the grazed system, where the goats and sheep are breed together. The multi-species grazing system allows the pathogenic agents to be spread among the different animal species. However, only 2 out of 70 sheep were seropositive by ELISA and none by PCR.

The current study was able to determine the genotype B by sequencing microsatellite region of MSP1a. This genotype was only previously reported twice and in cattle. The genotype B was associated to southern areas of Brazil by a phylogeographic clustering study (ESTRADA-PEÑA et al., 2009) and was detected in a sample from Minas Gerais (POHL et al., 2013). Due to the high degree of sequence variation, MSP1a sequences fail to provide phylogeographic origin information (DE LA FUENTE et al., 2005). Due to the high degree of sequence variation, MSP1a sequences fail to provide phylogeographic origin information (DE LA FUENTE et al., 2005). Although the phylogenetic analysis did not provide origin information, it provides phylogeographic information. The previous knowledge shows us that the genotype can be found in different regions of the country, but do not explain how it is transmitted.

The dissemination of genotypes among the country may be explained by cattle movement (QUIROZ-CASTAÑEDA; AMARO-ESTRADA; RODRÍGUEZ-CAMARILLO, 2016). The maintenance of the agent in the environment is not yet established, but the goats can may be acting as source of infection to the other species. Nevertheless, when distantly genotypes exist in the same area, the animal can be coinfected with multiples genotypes (PALMER et al., 2004).

The repeat F was found in a previous study conducted in the same property, where the samples were collected in 2014 (DA SILVA et al., 2018). In the present study, the samples were collected 4 years latter (2018) and the same repeat was also detected. The same strain may be maintained herein the herd by the movement interspecies. However, it is not possible to affirm once the strain could not be determine from the 2014 samples.

The strain F/91 found in the current study appears to have an intimate relation to the caprine species. This strain was never reported before in any animal species. The strain could not be identified in sheep and cattle. It is may due to the small sample size of cattle, not being reliable to say that the cattle in the property were not infected by the agent.

The bovine anaplasmosis can also be transmitted mechanically, such as by biting flies. In some areas, the mechanical transmission by biting flies can be more important than biological transmission, when some strains of *A. marginale* are unable to infect ticks, including *R. microplus* (RUIZ; PASSOS; BARBOSA RIBEIRO, 2005). However, it was not possible to collect those flies. Further studies are necessary to elucidate the transmission by flies.

Conclusion

To better evaluate the epidemiology of *A. marginale* in the farm from Paraiba state, Brazil, the study was conducted. A previous report showed the prevalence of *A. marginale* in goats collected in 2014 at the same property (DA SILVA et al., 2018). That was the first molecular report of *A. marginale* in small ruminants in Brazil.

The current study showed a greater prevalence in goats (2/119 by PCR and 49/119 by ELISA) when compared to bovine (0/5 by PCR and ELISA) and sheep (0/70 by PCR and 2/70 by ELISA). The genotype B and strain F/91 were detected by *masp1α* sequencing. The strain found in the study may be specific to goats and may maintained in the herd since 2014. However, only the repeat F was detected in the collected samples in 2014 (DA SILVA et al., 2018). Tthe higher prevalence found by ELISA when compared to PCR shows that the animals were chronic infected by *Anaplasma* spp., but by the serological assay used is not possible to determine the species found.

Further studies with a higher sample size of bovines, ticks and flies may be important to better elucidate the epidemiology of the agent or agents in the property.

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5. CONCLUSION

Little is known about the role of small ruminants in the epidemiology of *A. marginale*. Our team reported the first molecular report about infection of this agent in goats. Only another study, in Iran, found the bacterium in small ruminants. Despite the first insight about the infection of *A. marginale* in goats, few is known about its epidemiology. The genotybe B and strain F/91 were detected. This study showed that prevalence by serological and molecular means for cattle and sheep samples from this region were low or none for *A. marginale*, supposing a few hypotheses about the infection i) The *A. marginale* strain genotype may be specific for goats, meaning that this strain is not infectible to sheep and cattle. However, the cattle sample size was to low, being not reliable to affirm; ii) Climatic conditions found at the time of collection disfavor the growth of ticks in the area, thus limiting the transmission competence of the pathogen by these vectors scarce in the environment. Nevertheless, the presence of flies was observed in the farm, which can act as mechanical vector to the agent; iii) The animals were chronic infected, once the prevalence of ELISA-positive were greater than PCR-positive.