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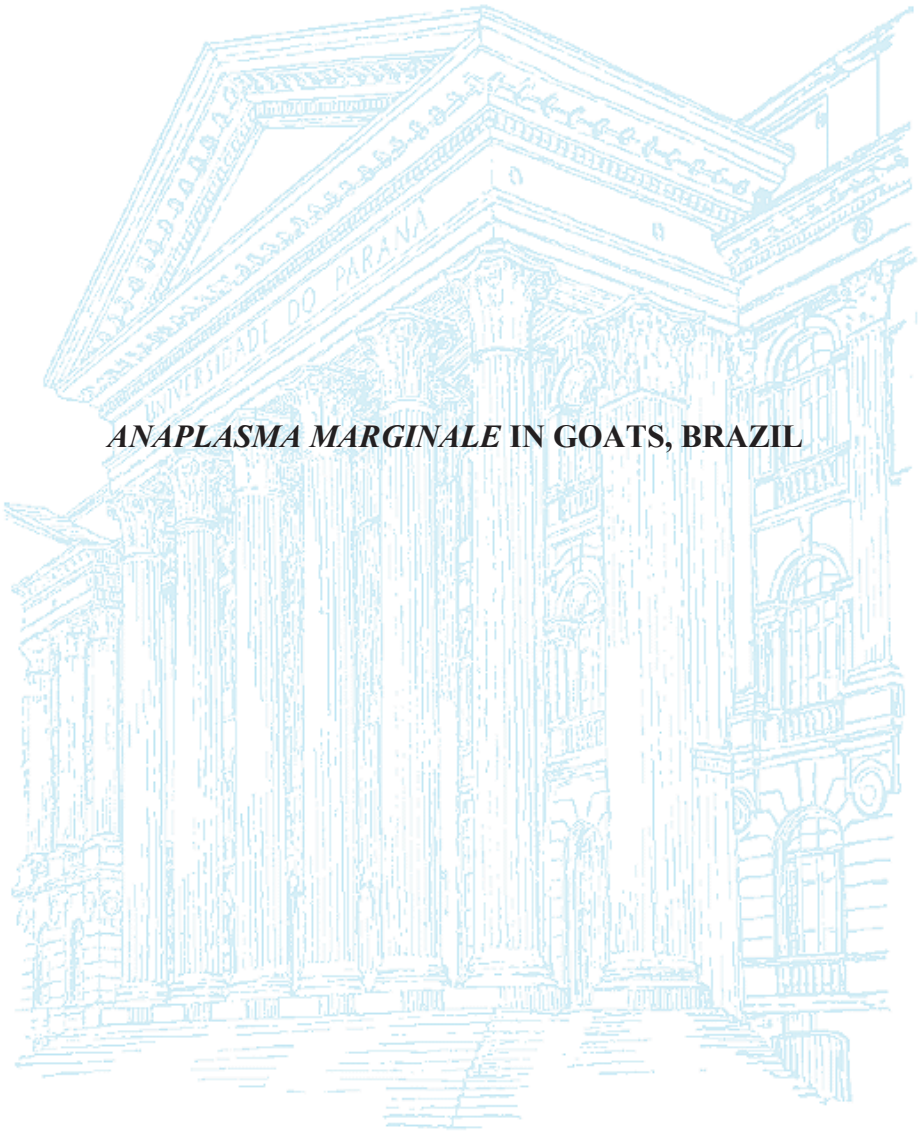
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS



ANAPLASMA MARGINALE IN GOATS, BRAZIL

Curitiba

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NAYARA BEZERRA DA SILVA

ANAPLASMA MARGINALE IN GOATS, BRAZIL

Dissertation presented as a partial requirement to obtain a Master's Degree in Veterinary Science, at the Graduate College in Veterinary Sciences, Sector of Agricultural Sciences, Universidade Federal do Paraná.

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Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em CIÊNCIAS VETERINÁRIAS da Universidade Federal do Paraná foram convocados para realizar a arguição da Dissertação de Mestrado de **NAYARA BEZERRA DA SILVA**, intitulada: "**ANAPLASMA MARGINALE INFECTION IN GOATS, NORTHEASTERN BRAZIL**", após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.
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CURITIBA, 08 de Dezembro de 2017.

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Avaliador Externo (USDA-USA)

I dedicate this work to my mother and father

“Honra teu pai e tua mãe, para que se prolonguem os teus dias na terra que o Senhor, teu Deus, te dá.”

- EX 20:12

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RESUMO

Anaplasma marginale é uma bactéria intraeritrocítica obrigatória do gênero *Anaplasma*, conhecida por causar anaplasmose bovina. Encontra-se distribuída em todo o mundo e causa grandes perdas econômicas nas indústrias de carne bovina e láctea. *A. marginale* foi descrita em muitas espécies. No entanto, os estudos envolvendo o diagnóstico sorológico de *A. marginale* em pequenos ruminantes são escassos. Até o presente momento, esta bactéria nunca foi detectada molecularmente em caprinos (*Capra hircus*). Assim, este estudo teve como objetivo estimar a prevalência de *A. marginale* e fatores associados à infecção em caprinos do Estado da Paraíba, no Nordeste do Brasil. O DNA de amostras de sangue de caprinos foi extraído e avaliado por uma reação em cadeia da polimerase convencional (cPCR) para a detecção da proteína de superfície 4 (major surface protein 4, *msp4*) de *A. marginale*. As amostras positivas foram posteriormente submetidas a cPCR para os genes *msp5* e *msp1α* de *A. marginale* e sequenciados pelo método de Sanger. Onze de 403 cabras (2,73%; IC 95%: 1,53-4,82%) foram positivas para o gene *msp4* de *Anaplasma*. O sequenciamento do gene *msp5* revelou a presença de *A. marginale* sensu stricto. Os caprinos infestados por carrapatos foram seis vezes mais propensos a estarem infectados com *A. marginale* ($P = 0,02788$). *Amblyomma parvum* (49/52, 94,23%) e *Rhipicephalus microplus* (3/52, 5,77%) foram as espécies de carrapatos identificadas parasitando os animais. Todas os caprinos positivos para *A. marginale* foram encontrados em fazendas com pastagem de múltiplas espécies ($P = 0,04$). O gene *msp1α* foi sequenciado encontrando o genótipo F nos animais estudados. Este é o primeiro relato molecular de infecção por *A. marginale* em caprinos. Além disso, descrevemos pela primeira vez o genótipo F no Brasil. Este estudo fornece a primeira informação sobre a infecção por *A. marginale* em cabras do Estado da Paraíba, no Nordeste do Brasil. Também demonstra que os caprinos podem desempenhar um papel na epidemiologia desta bactéria como um reservatório ainda não reconhecido. Carrapatos competentes que se alimentam de caprinos e bovinos podem transferir o patógeno entre as duas espécies de ruminantes.

PALAVRAS-CHAVE: anaplasmose bovina, cabras, genotipagem, pequenos ruminantes, PCR

ABSTRACT

Anaplasma marginale is an obligate intraerythrocytic bacterium in the genus *Anaplasma*, known for causing bovine anaplasmosis. It is distributed worldwide and causes extensive economic losses in the beef and dairy industries. *A. marginale* has been described in many species; however, studies involving the diagnosis of *A. marginale* in small ruminants are scarce. To date, this bacterium has never been molecularly detected in goats (*Capra hircus*). Thus, this study aimed to estimate the prevalence of *A. marginale* and factors associated with the infection in goats from the State of Paraíba, northeastern Brazil. DNA from goat blood samples were extracted and screened by a conventional PCR (cPCR) assay for the detection of *A. marginale* major surface protein 4 (*msp4*). Positive samples were further submitted to cPCR assays for *A. marginale msp5* and *msp1α* genes, and sequenced by Sanger method. Eleven out of 403 goats (2.73%; CI 95%: 1.53-4.82%) were positive for the *Anaplasma msp4* gene. Sequencing of the *msp5* gene revealed the presence of *A. marginale* sensu stricto. Tick-infested goats were six times more likely to be infected with *A. marginale* ($P = 0.02788$). *Amblyomma parvum* (49/52, 94.23%) and *Rhipicephalus microplus* (3/52, 5.77%) were the tick species identified feeding on the goats. All *A. marginale*-positive goats were found on farms with multispecies grazing ($P= 0.04$). The *msp1α* gene was sequenced and found the *A. marginale* genotype F in studied infected goats. This is the first molecular report of *A. marginale* infection in goats. Additionally, we describe for the first time the genotype F in Brazil. This study gives the first insight into *A. marginale* infection in goats from Paraíba State, northeastern Brazil and demonstrates that goats may play a role in the epidemiology of this bacterium as a yet unrecognized reservoir. Competent ticks feeding on goats and cattle may transfer the pathogen between the two livestock species.

KEY WORDS: bovine anaplasmosis, goats, genotyping, small ruminants, PCR

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FIRST REPORT OF *ANAPLASMA MARGINALE* INFECTION IN GOATS, BRAZIL

LIST OF ABBREVIATIONS

CAT – card agglutination test

cELISA - competitive Enzyme-linked Immunosorbent Assay

CF - complement fixation

cPCR – Conventional polymerase chain reaction

ELISA - Enzyme-linked Immunosorbent Assay

HVRs -central hypervariable regions

IFA - indirect fluorescent antibody

iELISA – indirect ELISA

Mab - Monoclonal antibody

MSP - Major Surface Protein

nPCR – nested PCR

PCR – Polymerase chain reaction

qPCR – quantitative PCR

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1. INTRODUCTION

Anaplasma marginale is an obligate intraerythrocytic bacterium in the genus *Anaplasma*, known for causing bovine anaplasmosis (AUBRY; GEALE, 2011). It is widely distributed in tropical, subtropical and temperate areas worldwide and causes extensive economic losses in beef and dairy industries (KOCAN et al., 2010b). In tropical and subtropical regions, *Rhipicephalus microplus* is the vector of bovine anaplasmosis (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). Despite host specificity of *R. microplus* for cattle, this tick species may be found parasitizing small ruminants (MA et al., 2016). In Brazil, *R. microplus* is endemic and hampers livestock production resulting in annual economic losses estimated at U$ 3.24 billion (GRISI et al., 2014).

Several methods may be employed in the diagnosis of *A. marginale*, such as Giemsa stained blood smears, serological and nucleic-acid-based assays. *A. marginale* infects the erythrocytes of its vertebrate hosts (AUBRY; GEALE, 2011). Following infection, infected cells are phagocytized by the host's reticuloendothelial system, resulting in mild to severe anemia and icterus without hemoglobinemia or hemoglobinuria and may be followed by fever, weight loss, lethargy, icterus, abortion, and often death in animals ≥ 2 years old (KOCAN et al., 2010b). Cattle that survive acute disease develop persistent infection and became reservoirs of *A. marginale*. Antigenic variations of the outer membrane proteins allow *A. marginale* to evade the host's immune system and maintain persistent infection (KOCAN et al., 2010b).

Many geographical isolates of *A. marginale* have been identified and differ in biology, morphology, protein sequences, antigenicity, and transmissibility by ticks. Six Major Surface Proteins (MSPs) have been described and are involved in host-pathogen interactions. The MSPs contain multiple B- and T-cell epitopes required for the development of protective immunity (KOCAN et al., 2004). Many risk factors have been associated to *A. marginale* infection, which has also been described in wild animals; however, their importance in the epidemiology of this pathogen is not fully comprehended (KOCAN et al., 2010b).

In northeastern Brazil, multispecies grazing is a common family subsistence practice on smallholder farms possibly facilitating interspecies transmission of pathogens (COSTA et al., 2008). *A. marginale* infection has been previously molecularly described in sheep (JALALI et al., 2013; YOUSEFI et al., 2017). However, to the best of our knowledge this bacterium has never been detected in goats. Thus, studies on *A. marginale* in goats are needed, particularly on those co-grazed with cattle, to better elucidate the role of other animal species in the epidemiological cycle of this bacterium.

2. OBJECTIVES

2.1. Objectives

- Determine the prevalence of *A. marginale* and factors associated with the infection in goats from the State of Paraíba, northeastern Brazil.

2.2. Specific objectives

- To screen goat blood samples for the presence of *A. marginale* infection by cPCR assays.
- To molecularly characterize *A. marginale msp1α, msp4* and *msp5* genes.
- Sampling and identification of ticks feeding on goats.
- To identify factors associated with *A. marginale* infection.

3. BOVINE ANAPLASMOSIS IN BRAZIL – A REVIEW

3.1. Introduction

Anaplasma marginale is an obligate intraerythrocytic bacterium in the genus *Anaplasma*, known for causing bovine anaplasmosis (AUBRY; GEALE, 2011). *A. marginale* appears on or near the margin of the infected erythrocyte as dense rounded bodies, with approximately 0.3–1.0 μm in diameter (OIE, 2015). It was first described in 1910 by Arnold Theiler who found small inclusions in the erythrocytes of sick cattle in Africa (THEILER, 1910). *A. marginale* is widely distributed in tropical, subtropical and temperate regions worldwide, occurring in six continents (KOCAN et al., 2010b; KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015), and causing extensive economic losses in the beef and dairy industries (KOCAN et al., 2010b).

In vertebrate hosts, *A. marginale* incubation period may vary from 7-60 days (KOCAN et al., 2010b). During acute infection, ≥ 70% of erythrocytes may be parasitized and bacteremia may exceed 10^9 organisms per mL (PALMER; BROWN; RURANGIRWA, 2000). Following infection, infected cells are phagocytized by the host's reticuloendothelial system, resulting in mild to severe anemia and icterus without hemoglobinemia or hemoglobinuria, and may be followed by fever, weight loss, lethargy, abortion, and often death of animals ≥ 2 years old (KOCAN et al., 2010a).

Cattle that survive acute disease develop persistent infection, characterized by cyclic bacteremia (10^2–10^7 organisms per mL of blood) and became reservoirs for *A. marginale* infection (PALMER; BROWN; RURANGIRWA, 2000). Persistently infected cattle are crucial for *A. marginale* perpetuation as they are a source of organisms for transmission. These carrier animals show lifelong immunity and, upon homologous challenge, do not develop clinical disease (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). Antigenic variations of the outer membrane proteins, by recombinatorial mechanisms, allow *A. marginale* to evade the host's immune system and maintain persistent infection. Multiple *A. marginale* strains may simultaneously infect a host, known as a superinfection (QUIROZ-CASTAÑEDA; AMARO-ESTRADA; RODRÍGUEZ-CAMARILLO, 2016).

Anaplasma marginale is a vector-borne disease mainly transmitted by ticks of the genus *Dermacentor* and *Rhipicephalus* (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). Development within ticks initiates after the blood-meal, and is a complex process involving different morphological stages and tick tissues. *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. Subsequently, *A. marginale* invades other tissues, mainly the salivary glands, and may be efficiently transmitted (BRAYTON, 2012). Biological transmission has been shown to be more efficient than mechanical transmission (DE LA FUENTE et al., 2010). However, in areas where the vector is absent or in cases of strains that are not tick-transmissible, transmission by bloodsucking arthropods or iatrogenic transmission may be crucial (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015).

Transmission of *A. marginale* may occur interstadially and intrastadially by one- and three-host ticks (KOCAN et al., 2010a). Male ticks may be very important in the epidemiology of *A. marginale*, since they are intermittent feeders, show longevity under favorable natural conditions and may became persistently infected by *A. marginale* (KOCAN et al., 2010a). These characteristics make male ticks efficient *A. marginale* vectors, particularly for one-host ticks (KOCAN et al., 2010a). Transovarial transmission does not seem to occur (DUMLER et al., 2001). In addition, previous studies have shown 5.9% to 41% of *A. marginale*-positive calves born from chronically infected dams, showing that transplacental transmission may be important in the maintenance of *A. marginale*-infected herds (COSTA et al., 2016; GRAU et al., 2013; SILVA et al., 2015a; SILVA; ANDRÉ; MACHADO, 2016; SILVA; CASTRO; FONSECA, 2014; SILVESTRE et al., 2016).

Several methods may be employed in the diagnosis of *A. marginale*. Giemsa stained blood smears are used to detect intraerythrocytic organisms, particularly during the acute phase of the disease (AUBRY; GEALE, 2011), detecting levels of $>10^6$ infected erythrocytes per mL (TORIONI DE ECHAIDE et al., 1998). However, it lacks sensitivity and may not be reliable in the determination of chronic or subclinical cases, and in pre-symptomatic or in persistently infected animals (AUBRY; GEALE, 2011). Nucleic-acid-based tests are highly sensitive and specific, detecting very low levels of bacteremia (AUBRY; GEALE, 2011). However, some of these techniques may be expensive, differ among laboratories, have significant quality control problems and are not practical when evaluating a large number of animals (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015; OIE, 2015).

Serological tests are more suitable for diagnosis in chronic cases and are most efficient in serosurveys, especially when testing a large herd (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015; TORIONI DE ECHAIDE et al., 1998). A wide variety of assays have been developed, such as complement fixation (CF) test, capillary agglutination assay, and indirect fluorescent antibody (IFA) test. However, these assays lack sensitivity and specificity and may show non-specific results (AUBRY; GEALE, 2011). Currently, a competitive enzyme-linked immunosorbent assay (cELISA), that uses recombinant MSP5 antigens and MSP5-specific

monoclonal antibody (MAb), has proven very sensitive and specific for the detection of *Anaplasma*-infected animals (CHUNG et al., 2014). Nevertheless, previous studies have shown that cross-reactivity may occur with antibodies from other *Anaplasma* species, such as *A. ovis, A. centrale* and *A. phagocytophilum* (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). Card agglutination test (CAT) is also recommended for identifying infected animals, as it is sensitive, non-time-consuming and may be performed at field conditions. Like other serological tests, nonspecific reactions may occur and the assay's interpretation may also be a varying factor in the result (OIE, 2015).

Geographical isolates of *A. marginale* differ in biology, morphology, protein sequences, antigenicity, and transmissibility by ticks (KOCAN et al., 2004). *A. marginale* has been characterized based on its outer membrane proteins (DE LA FUENTE et al., 2005; QUIROZ-CASTAÑEDA; AMARO-ESTRADA; RODRÍGUEZ-CAMARILLO, 2016). Six major surface proteins (MSPs) are mainly involved in host-pathogen interactions, and evolve more rapidly due to positive selective pressures exerted by the host's immune system (DE LA FUENTE et al., 2010). MSP1a, MSP4, MSP5 are encoded by single genes, while multi-gene families encode the MSP1b, MSP2 e MSP3 proteins (ALLEMAN et al., 1997; BRAYTON et al., 2005; OBERLE; PALMER; BARBET, 1993; PALMER et al., 1994; VISSER et al., 1992). The MSPs contain multiple B- and T-cell epitopes required for the development of protective immunity and may be responsible for cross-protection in different *A. marginale* strains (MOLAD et al., 2004).

MSP1a and MSP1b are part of the MSP1 complex, composed of two structurally unrelated polypeptides (MACMILLAN et al., 2006). MSP1a is involved in the adhesion of *A. marginale* to bovine erythrocytes and tick cells, being imperative for the infection of this pathogen, while MSP1b is an adhesin only for bovine erythrocytes (GARCIA-GARCIA et al., 2004). Its encoding gene, *msp1α*, is a stable genetic marker, conserved during the bacteria's developmental cycle in the hosts, and with no variation within a strain (DE LA FUENTE et al., 2005).

Geographical strains may be differentiated based of the molecular weight of the MSP1a protein since it varies in size between *A. marginale* isolates, due to the different numbers (23–31) of constitutional amino acid tandem repeats (CABEZAS-CRUZ et al., 2013). MSP1a contributes to immunity development against challenge with homologous and heterologous *A. marginale* strains (CABEZAS-CRUZ et al., 2013). High genetic heterogeneity of the MSP1a tandem repeats may be found in endemic areas. On those areas, multiple *A. marginale* strains may coexist, which seems to be associated to worldwide cattle movement and

independent transmission events (CABEZAS-CRUZ et al., 2013; PALMER; RURANGIRWA; MCELWAIN, 2001).

In Brazil, 62 different MSP1a tandem repeat sequences have been reported, in the States of Minas Gerais (DE LA FUENTE et al., 2002, 2004; POHL et al., 2013; SILVESTRE et al., 2016), Paraná (VIDOTTO et al., 2006), Rio de Janeiro (BAÊTA et al., 2015; SILVA et al., 2015a, 2014b), Pará (SILVA et al., 2014a), Goiás and São Paulo (MACHADO et al., 2015). The MSP1a tandem repeat sequences are summarized on Table 1. Sequence analysis of the MSP1a tandem repeats has shown the genetic heterogeneity of *A. marginale* strains in Brazil, which is a characteristic of endemic regions (MACHADO et al., 2015; PALMER; RURANGIRWA; MCELWAIN, 2001; POHL et al., 2013).

MSP2 and MSP3 are highly conserved immunodominant proteins (BRAYTON et al., 2003, 2005). Antigenic variation of both proteins occurs during rickettsemic peaks in order for the new emergent variants to evade the host's existing immune response, contributing to the maintenance of infection (BRAYTON et al., 2003, 2005). *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., 2003, 2005).

MSP4 is a highly conserved protein with an unknown function. However, the study of its encoding gene, *msp4*, has been widely used in phylogeographic studies due to its sequence variation (DE LA FUENTE et al., 2002). The immunodominant protein MSP5 is highly conserved with unknown function. It is mainly used as an antigen in serologic diagnostic (KOCAN et al., 2003).

3.2. Prevalence

In Brazil, climatic factors and environmental conditions vary greatly throughout the country and influence *A. marginale* occurrence. Thus, the epidemiological situation of *A. marginale* countrywide may be characterized as enzootically unstable, enzootically stable and free (SOTT et al., 2016).

In areas of enzootic instability, climatic conditions may affect tick development and survival during certain periods of the year, causing the reduction or eradication of these parasites (SOTT et al., 2016). In an unstable area, prevalence is < 75%, since, in these areas the majority of the animals are not infected during the first months of life and lack in immune response; thus, leaving these animals susceptible to acute infection, with high mortality rates (AUBRY; GEALE, 2011; MAHONEY et al., 1972). Enzootic instability may happen during

certain times of the year when conditions are favorable to tick growth, which may result in outbreaks (AMORIM et al., 2014).

In enzootically stable areas, climatic conditions allow for tick growth and persistence in the environment, which may lead to high *A. marginale* prevalence (≥ 75%), but with rare clinical disease (MAHONEY; ROSS, 1972). Enzootic stability is usually present in tropical and subtropical areas where calves become infected very soon after birth, and while still carrying maternal antibodies that provide immunological protection (AMORIM et al., 2014). In those areas, *A. marginale* is present throughout the year, and offering a constant immunological challenge that may result in high antibody levels and protection against clinical disease (COSTA et al., 2011).

Anaplasma marginale free areas have unfavorable climatic conditions for development of the tick vector and consequently, the development of the pathogen (AMORIM et al., 2014). Animals in these areas are highly susceptible to infection as they may lack in antibodies (COSTA et al., 2011). In Brazil, the States of Acre (BRITO et al., 2010), Bahia (ARAÚJO et al., 1998; BARROS et al., 2005; COSTA et al., 2016), Goiás (SANTOS; MADRUGA; LINHARES, 2001), Piauí (SOUZA et al., 2013) and Rondônia (BRITO et al., 2010), are reported as enzootically stable. While unstable areas may be found in the States of Mato Grosso (SILVA et al., 2015c), Santa Catarina (CANEVER et al., 2014), Sergipe (OLIVEIRA; PEDREIRA; ALMEIDA, 1992) and Pernambuco (SANTOS et al., 2017).

Some areas in Brazil may be both enzootically stable and unstable, during different periods of the year, such as in Mato Grosso do Sul (MADRUGA et al., 2000; MADRUGA; AYCARDI; PUTT, 1983; MELO et al., 2001; PEREIRA; GUIMARÃES; ROCHA, 2009; SILVA et al., 2006), Minas Gerais (BARBIERI et al., 2016; CARVALHO et al., 2012; POHL et al., 2013; RIBEIRO et al., 1984), Pará (SILVA et al., 2015b, 2014c), Paraíba (COSTA et al., 2011, 2013; COSTA; SIMÕES; RIET-CORREA, 2009; SOUZA et al., 2001), Paraná (ANDRADE et al., 2001; MARANA et al., 2009; SOTT et al., 2016; VIDOTTO et al., 1998; YOSHIHARA et al., 2003), Rio de Janeiro (SILVA et al., 2015b) and Tocantins State (SILVA et al., 2015c; TRINDADE et al., 2011).

In northern Brazil, the molecular prevalence of *A. marginale* ranged from 92.4% to 98.6% by a PCR for the *msp5* gene (BRITO et al., 2010). In Pará State, previous studies have shown seroprevalences of 68.3% (112/164) and 74.5% (506/ 679) by indirect ELISA (iELISA) (GUEDES JUNIOR et al., 2008; SILVA et al., 2014c). Two varying prevalence results were found in Tocantins State through iELISAs of 52.6% and 89.9%, which shows that areas of

enzootic stability and instability may be present in different parts of the State (SILVA et al., 2015c; TRINDADE et al., 2011).

In the northeastern region of Brazil, the reported seroprevalence by ARAÚJO et al. (1998) was 97.2% (105/108) by ELISA, 97.2% (105/108) by IFAT and 90.7% (98/108) by CAT in Bahia State. While BARROS et al. (2005), COSTA et al. (2016) and MADRUGA et al. (2000) reported 97.8%, 69.1% (65/94) and 96.9% (314/324) by ELISA, respectively. Seroprevalence by IFAT was 97.2% (315/324) (MADRUGA et al., 2000). In Paraíba, seroprevalence by IFAT was 16.5% (42/255) (COSTA et al., 2013), 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 (OLIVEIRA; PEDREIRA; ALMEIDA, 1992) by CAT. AMORIM et al. (2014) found in Ibicaraí, Bahia, prevalence of 31.1% (96/309) by blood smear, however, when the same samples were analyzed by nested PCR (nPCR) of the *msp5* gene, it resulted in 63.1% (195/ 309) prevalence. Difference in the results are most likely because nPCR is more sensitive when compared to direct blood smear analysis (AUBRY; GEALE, 2011). This study also showed that tick infestation and age (≤9 months old) were important risk factor (AMORIM et al., 2014). Also in Ibicaraí, COSTA et al. (2016) found similar prevalence by iELISA, 69.1% (65/94), but only 30.8% (37/120) were positive by nPCR of the *msp1α* gene. Analysis of bovine anaplasmosis outbreaks in different areas of the Sertão region, Paraíba State, resulted in low suggestive prevalence (8.5% (13/153) by blood smear analysis with high parasitemia, 1.8-80% (COSTA et al., 2011). Low infection prevalence might be associated to the low sensitivity of blood smear diagnostics (KOCAN et al., 2010b). However, associated to the outbreaks were factors such as rise in rainfall levels, presence of flies and ticks on the animals and properties, inappropriate acaricide application practices, age (older animals) and introduction of new tick infected cattle into the herds. All factors that have been previously associated to *A. marginale* outbreaks (COSTA et al., 2011). Consistent with findings in other northeastern regions of Brazil studies using blood smear found low levels of infection, 12.3% (319/2593) and 96/309 (31.10), in cattle from Bahia and Sergipe (AMORIM et al., 2014; OLIVEIRA; PEDREIRA; ALMEIDA, 1992).

In the Midwest, SANTOS; MADRUGA; LINHARES, (2001) found in Goiás *A. marginale* prevalence of 99.2% (517/521) and 96.9 (505/521) by ELISA and IFAT, respectively. Also in Goiás State, 50 animals with clinical signs, which included fever, anemia and reduction in milk production were studied by MACHADO et al. (2015), who compared four different diagnostics techniques, resulting in 34% (17/50) by blood smear, 50% (25/50) by IFAT, 54% (27/50) by ELISA and 38% (19/50) by quantitative PCR (qPCR) of the *msp*1α gene.

The ELISA showed 92.6% agreement with the IFAT. The study also showed that when compared, calves, heifers, pregnant and lactating cows, the heifers and calves were four and three times, respectively, more likely to die from anaplasmosis than lactating cows. Furthermore, prior to the outbreak the animals were transferred from a tick free to a tick infested area, where they became parasitized. Additionally, a high number of flies were reported on the property (MACHADO et al., 2015). In the State of Mato Grosso do Sul seroprevalence by CAT, CF and ELISA were 100% (50/50) (MADRUGA et al., 1985) 7.95% (53/667) (MADRUGA; AYCARDI; PUTT, 1983) and 43.9% (56/128) (SILVA et al., 2006), respectively, in the investigated animals. In Mato Grosso State SILVA et al., (2015) reported 41.1% (212/516) by iELISA.

In Minas Gerais State prevalence by IFAT varies 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). By CAT, results were 73.5% (233/317) (RIBEIRO; REIS, 1981; SILVEIRA et al., 2014). A study in the mesoregion of Zona da Mata, Minas Gerais State, suggests a 35% (14/40) prevalence by blood smear (PAULA et al., 2015), however, serological studies in the same region shows higher results, namely IFAT and CAT, 81.1% (257/317) and 73.5% prevalence (233/317), respectively (RIBEIRO et al., 1984). Through microscopic examination of blood smears POHL et al. (2013) detected suggestive *A. marginale* infection in 48% (48/100) of the studied cattle, with bacteremia ranging from 0.1 to 16.1%. In the same study, results obtained by qPCR revealed 70.2% (66/94) positivity, showing that this method was more sensitive than direct examination of blood smears (POHL et al., 2013). A study in the microregion of Caparaó, Espírito Santo State, showed suggestive prevalence by means of blood smear analysis of 17.6% (32/182) (JUNIOR et al., 2008).

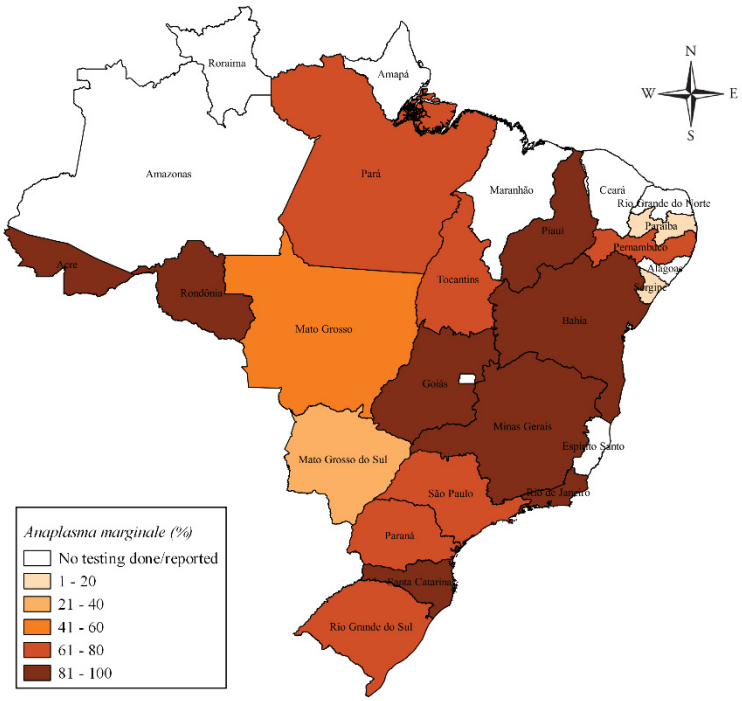
In Rio de Janeiro State seroprevalence ranged from 31.4% (13/41) to 98,21% (219/223) by iELISA (SILVA et al., 2015b; SOUZA et al., 2000; SOUZA et al., 2001). Studies in Rio de Janeiro State show that tick infestation, breed, production type (milk/beef), age and stock density may all promote *A. marginale* infection (SILVA et al., 2015b; SILVA; CASTRO; FONSECA, 2014). In addition, during the cows reproductive cycle it was observed that pregnancy and lactation influenced significantly ($p<0.05$) in the seropositivity of the animals, especially the cows that were lacting for the first time; however, the seroprevalence dropped during peripartum (SILVA; FONSECA, 2013).

In a serosurvey in São Paulo State SILVA; ANDRÉ; MACHADO, (2016) reported 80% (16/20) prevalence by both ELISA and IFAT. During an outbreak in the municipality of Lins (State of São Paulo), MACHADO et al. (2015) found low seropositivity by ELISA and

IFAT, 58% (29/50) and 52% (26/50), respectively. These results could be associated to the low or negative antibodies levels during the acute phase of the disease, making serological diagnostics unreliable during this period (AUBRY; GEALE, 2011). Conversely, during acute anaplasmosis high levels of rickettsemia (> 109 mL-1) are usually present (PALMER; BROWN; RURANGIRWA, 2000), facilitating the use of direct and molecular diagnostics. MACHADO et al. (2015) found higher results positivity by blood smear and by *msp1α* gene PCR, 84% (42/50) and 94% (47/50), respectively. The PCR results are in agreement with SILVA; ANDRÉ; MACHADO (2016) that found 100% (20/20) positivity based on the *msp1α* gene analysis. Contrary to findings by MACHADO et al. (2015), GONÇALVES et al. (2011) found, in the medical records from the Veterinary Hospital at the Paulista State University, in Botucatu, low prevalence from blood smear analysis. The results show that 31.5% (361/1147) of the bovine patients that received medical care at the hospital were positive for *A. marginale* infection (GONÇALVES et al., 2011). Additionally, they found that mortality rates were higher in animals between 2-6 months (24.7% (283/1147) and that the disease increased during the fall; also, European (*Bos taurus)* cattle and crossbred animals, with moderate to high tick infestation showed higher infection rates (GONÇALVES et al., 2011). Furthermore, clinical signs included lethargy, drop or lack of appetite, dehydration, weight loss, pale mucous membranes, weakness, dry brittle coat, tachycardia, hyperthermia, dyspnea, hemoglobinuria, icterus and gastrointestinal stasis (GONÇALVES et al., 2011).

In Paraná State, different serological diagnostic techniques were used in serosurveys resulting in 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 et al., 2016) by iELISA; 77.7% (267/344) (SOTT et al., 2016) by ELISA; and 67.4% (281/417) (VIDOTTO et al., 1997) by IFAT, prevalence. ARTILES et al. (1995) described a 64% (794/1246) prevalence by CAT in Rio Grande do Sul State. Seroprevalence in Santa Catarina State was reported as 86% (516/600) by IFAT (DALAGNOL; MARTINS; MADRUGA, 1995). A study in Ponte Alta Municipality, Santa Catarina State, showed prevalence of 60.6% (20/33) by *msp1α* gene multiplex PCR, during an outbreak in a beef herd (CANEVER et al., 2014). Age did not seem to be a factor in the outbreak, since the animals varied from 8-48 months. However, *R. microplus* ticks were reported on the properties, and could be related to the infections, since this tick species is a known vector of *A. marginale* (CANEVER et al., 2014). The geographical distribution of *A. marginale* spp. in cattle in Brazil by serologic and molecular methods is illustrated in Figure 1.

Figure 1. Geographical illustration of serologic and molecular occurrence of *Anaplasma marginale* in cattle in Brazil based on data from Table 2.



3.3. Risk Factors

Many risk factors have been associated to *A. marginale* infection, such as vector population, age, seasonal variation and breed. Ticks are the biological and most efficient vectors for *A. marginale* and their occurence has been associated with high *A. marginale* prevalence (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015). Low seroprevalence has been shown on properties with low tick infestation. In addition higher seroprevalence was described when acaricide applications was infrequent, suggesting a higher cattle exposure to vector (COSTA et al., 2013). In areas where the vector is absent or in cases of strains that are not transmissible by ticks, transmission by bloodsucking arthropods may be crucial (KOCAN; DE LA FUENTE; CABEZAS-CRUZ, 2015).

Animals that are constantly exposed to *A. marginale* infection, especially during the first nine months of life, develop immunity becoming resistant to clinical disease. Calves younger than nine months of age carry maternal antibodies, which confers them more resistance to acute *A. marginale* infection (MAHONEY; ROSS, 1972). In enzootically unstable areas, the animals are not exposed to *A. marginale* infection early in life and are left highly susceptible to

clinical infection. In seronegative adult cattle, ≥ 2 years old, infection signs are usually more severe, and may lead to death (KOCAN et al., 2010b).

Anaplasma marginal infection is highly influenced by seasonal variations. Outbreaks have been described in areas where the environment is humid, such as during rainy seasons or in artificially irrigated areas, in which the climate is more favorable to the survival of the tick vector (COSTA; SIMÕES; RIET-CORREA, 2009). A study has reported the occurrence of *A. marginale* outbreaks during periods of high rainfall that coincided with an increase in the number of ticks and biting flies (COSTA et al., 2011). However, outbreaks have also been described in drier regions where ticks do not survive periods of drought, but are introduced at the beginning of the rainy season and can persist and reproduce in the environment, resulting in clinical disease (COSTA; SIMÕES; RIET-CORREA, 2009).

Breed might be involved in positivity for *A. marginale.* A study in Brazil shows that European breeds (*Bos taurus*) may be more susceptible to *A. marginale* infection than Zebu cattle (*Bos indicus*) and its crossbreeds, as the *Bos Taurus* cattle showed high levels of infection (GONÇALVES et al., 2011). In addition, seropositivity may be higher during different moments of the cows' reproductive cycle, such as pregnancy and lactation (SILVA; FONSECA, 2013). Gender does not seem to be a risk factor for *A. marginale* positivity (SANTOS et al., 2017; SOUZA et al., 2000).

3.4. Wildlife

Anaplasma marginale infection has been described in different wild animals, although it is mainly known for infecting domestic ruminants. In Brazil, free-living pampas deer (*Ozotoceros bezoarticus*) presented 38% (23/60) positivity by PCR (SILVEIRA et al., 2013), and 73.9% (13/23) of brown brocket deer (*Mazama gouazoubira*), 50% (1/2) of captive *M. gouazoubira* and 100% (4/4) of captive *Blastocerus dichotomus* (SILVEIRA; RABELO; RIBEIRO, 2012). Another investigation identified distinct genotypes found in Andean deer (*Hippocamelus antisensis*) and, surprisingly, also in giant anteater (*Myrmecophaga tridactyla*), a non-ruminant and neither herbivorous (GUILLEMI et al., 2016).

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Tabela 1. MSP1a repeat sequences in Brazil.

Region	Isolated from	Strain							No of repeats	References
MG	Cattle	Brazil	B	B	Q	σ	μ		5	(DE LA FUENTE et al., 2002)
		Brazil 9	α	β	τ	M			4	(DE LA FUENTE et al., 2004)
		Brazil 12	α	β	β	N			4	
		Brasil 5	C	F	N				3	
		Minas-1	τ	57	β	β	γ		5	(POHL et al., 2013)
		Minas-2	Is9	24	24	25	31		5	
		Minas-3	α	β	β	γ			4	
		Minas-4	B	Q	B	M			4	
		Minas-5	13	27	27	27			4	
		Minas-6	72	62	61				3	
		Minas-11	τ	57	13	18			4	
		Minas-13	α	β	β	13			4	
		UFMG-2	13	27	27				3	
		UFMG-1	13	42	13	18			4	
		UFMG-3	13	MGI19	MGI19				3	(SILVESTRE et al., 2016)
PR	Cattle	PR1	α	β	β	β	β	Γ	6	(VIDOTTO et al., 2006)
		PR2	λ	F	Ω	ω	ψ		5	
		PR3	τ	κ	θ				3	
RJ	Cattle/cell culture	AmRio1	162	F	17	F	F		5	(BAÊTA et al., 2015)
		AmRio2	α	β	β	β	F		5	
	Water buffalo	16	τ	10	10	15			4	(SILVA et al., 2014a)
		17	4	10	3				3	
		19	α	β	β	β	Γ		5	
		20	α	β	β	Γ			4	
		22	4	63	63				3	

	Tick	R. microplus 1	163	164	164	164	61		5	
		A. cajennense 1	165	13	18				3	
		A. cajennense 2	165	13	Γ				3	
	Cattle	Rio1a	78	24	24	25	31		5	(SILVA et al., 2015)
		Rio2a	4	63	27				3	
		Rio3a	τ	10	10	15			4	
		Rio4d	165	10	10	166			4	
		Rio5b	167	168	β	β	169	170	6	
		Rio6a	78	24	24	171	31		5	
		Rio8c	4	63	4				3	
		Rio9a	4	63	3				3	
		Rio9b	78	24	172	24	173		5	
		Rio9c	α	174	β				3	
		Rio9d	175	63	27				3	
		Rio10a	τ	10	10	176			4	
		Rio10b	174	176	β	β	τ		5	
		Rio15a	4	63	177				3	
		Rio15b	178	179	180	181	182		5	
		Rio16a	163	164	164	164	61		5	
		Rio17c	176	174	β	β	τ		5	
		Rio18c	182	24	183	184	185		5	
		Rio20b	174	176	β	β	186		5	
PA	Water buffalo	N/I	4	63	27				3	(SILVA et al., 2014b)
		N/I	78	24	24	25	31		5	
		N/I	τ	10	10	15			4	
		N/I	162	63	27				3	
SP	Cattle	Lins SP/7	τ	10	15				3	(MACHADO et al., 2015)
		Lins SP/11	191	13	18				3	

		Lins SP/12	α	β	β				3
		Lins SP/110	α	β	β	β	13		5
		Lins SP/703	α	β	β	192			4
		Lins SP/1136	τ	β	100				3
		Lins SP/1228	α	β	β	Γ			4
		Lins SP/1450	193	β	100				3
		Lins SP/1453	191	13	Γ				3
GO	Cattle	Mambaí GO/1017B	α	β	β	β	Γ		5
		Mambaí GO/1806B	E	F	φ	φ	F	F	6

Abbreviations: MG, Minas Gerais; PR, Paraná; RJ, Rio de Janeiro; PA, Pará; SP, São Paulo; GO, Goiás, N/I; not informed.

Table 2. Occurrence of *Anaplasma marginale* infection in Brazil.

Geographic Area	Population	No of cattle	Diagnostic methods	Occurrence (%)	References
Southeastern Brazil					
ES					
ES	Survey	182	Blood smear	32/182 (17.6)	(JUNIOR et al., 2008)
MG					
MG	Survey	865	Card agglutination test	770/865 (89)	(RIBEIRO; REIS, 1981)
Mata region	Survey	317	Card agglutination test	233/317 (73.5)	(RIBEIRO et al., 1984)
			IFAT	257/317 (81.1)	
Metalúrgica region	Survey	88	IFAT	81/88 (92)	(MELO et al., 2001)
Lavras	Survey	131	IFAT	123/131 (94)	(PEREIRA; GUIMARÃES; ROCHA, 2009)
Campo das Vertentes	Survey	337	IFAT	315/337 (93.5)	(CARVALHO et al., 2012)
Cordislândia	Survey	94	qPCR (msp1β gene)	66/94 (70.2)	(POHL et al., 2013)
Palma	Survey	40	Blood smear	14/40 (35)	(PAULA et al., 2015)
MG	Survey	400	IFAT	385/400 (96.2)	(BARBIERI et al., 2016)
SP					
Botucatu	Medical history	1147	Blood smear	361/1147 (31.5)	(GONÇALVES et al., 2011)
Lins	Outbreak	50	Blood smear	42/50 (84)	(MACHADO et al., 2015)
			ELISA	29/50 (58)	
			IFAT	26/50 (52)	
			qPCR (msp1α gene)	47/50 (94)	
Taiaçu	Survey	20	ELISA	16/20 (80)	(SILVA; ANDRÉ; MACHADO, 2016)
			IFAT	16/20 (80)	
			qPCR (msp1α gene)	20/20 (100)	
RJ					

RJ	Survey	532	Indirect ELISA	485/532 (91.1)	(SOUZA et al., 2000)
Baixada Fluminense	Survey	41	Indirect ELISA	13/41 (31.4)	(SILVA et al., 2015a)
Metropolitan region	Survey	22		17/22 (75)	(SILVA; CASTRO; FONSECA, 2014)
Southern Brazil					
PR					
Londrina	Survey	417	IFAT	281/417 (67.4)	(VIDOTTO et al., 1997)
Londrina	Survey	410	Competitive ELISA	359/410 (87.5)	(VIDOTTO et al., 1998)
Londrina	Survey	708	Competitive ELISA (rMSP5)	658/708 (92.9)	(ANDRADE et al., 2001)
Umuarama	Survey	226	Competitive ELISA	172/226 (76.1)	(YOSHIHARA et al., 2003)
PR	Survey	223	Competitive ELISA	131/223 (58.7)	(MARANA et al., 2009)
PR	Survey	90	Indirect ELISA	64/90 (71.1)	(ELIAS et al., 2016)
Realeza	Survey	344	ELISA	267/344 (77.7)	(SOTT et al., 2016)
RS					
Bagé	Survey	1246	Card agglutination test	794/1246 (64)	(ARTILES et al., 1995)
SC					
Lages	Survey	600	IFAT	516/600 (86)	(DALAGNOL; MARTINS; MADRUGA, 1995)
Ponte Alta	Outbreak	33	Multiplex PCR (msp1α gene)	20/33 (60.6)	(CANEVER et al., 2014)
Northern Brazil					
AC					
AC		225	PCR (msp5 gene)	208/225 (92.4)	(BRITO et al., 2010)
PA					
PA	Survey	246	Indirect ELISA	168/246 (68.3)	(GUEDES JUNIOR et al., 2008)
PA	Survey	679	Indirect ELISA	506/ 679 (74.5)	(SILVA et al., 2014)

PA	Survey	679	Indirect ELISA	506/679 (74.5)	(SILVA et al., 2015b)
RO					
RO	Survey	1650	PCR (msp5 gene)	1627/1650 (98.6)	(BRITO et al., 2010)
TO					
Araguaína	Survey	506	Indirect ELISA	455/506 (89.9)	(TRINDADE et al., 2011)
TO		508		267/508 (52.6)	(SILVA et al., 2015b)
Northeastern Brazil					
BA					
Jequié	Survey	86	ELISA	85/86 (98.8)	(ARAÚJO et al., 1998)
			IFAT	83/86 (96.5)	
			Card agglutination test	85/86 (98.8)	
Vitória da Conquista		119	ELISA	116/119 (97.5)	
			IFAT	115/119 (96.6)	
			Card agglutination test	114/119 (95.8)	
Itabuna		119	ELISA	113/119 (95)	
			IFAT	117/119 (98.3)	
			Card agglutination test	96/119 (80.7)	
BA	Survey	324	ELISA	314/324 (96.9)	(MADRUGA et al., 2000)
			IFAT	315/324 (97.2)	
BA	N/I	823	ELISA	814/823 (98.9)	(BARROS et al., 2005)
			ELISA (rMSP5)	798/823 (97)	
Ibicaraí	Survey	309	Blood smear	96/309 (31.1)	(AMORIM et al., 2014)
			nPCR (msp5 gene)	195/ 309 (63.1)	
Ibicaraí	Survey	94	Indirect ELISA	65/94 (69.1)	(COSTA et al., 2016)
		120	nPCR (msp1α gene)	37/120 (30.8)	

PA					
São José de Espinharas		120	Blood smear	7/120 (5.8)	(COSTA et al., 2011)
PB					
PB	Medical history	14	Medical charts	12/14 (85.7)	(COSTA; SIMÕES; RIET-CORREA, 2009)
Piancó	Outbreak	310	Blood smear	26/310 (8.4)	(COSTA et al., 2011)
Aparecida		287	Blood smear	11/287 (3.8)	
Pombal		20	Blood smear	8/20 (40)	
Patos		27	Blood smear	12/27 (44.4)	
PB	Survey	509	IFAT	84/509 (16.5)	(COSTA et al., 2013)
PB	Survey	223	Indirect ELISA	219/223 (98.2)	(SOUZA et al., 2001)
PE					
Petrolina	Survey	468	IFAT	275/468 (58.8)	(SANTOS et al., 2017)
Ouricuri		393		305/393 (77.6)	
PI					
PI	Survey	202	IFAT	180/202 (89.1)	(SOUZA et al., 2013)
			PCR	154/202 (76.2)	
SE					
SE	Survey	2593	Blood smear	319/2593 (12.3)	(OLIVEIRA; PEDREIRA; ALMEIDA, 1992)
			Card agglutination test	423/2593 (16.3)	
Midwest					
GO					
Goiânia	Survey	521	ELISA	517/521 (99.2)	(SANTOS; MADRUGA; LINHARES, 2001)
			IFAT	505/521 (96.9)	
Mambaí		50	Blood smear	17/50 (34)	(MACHADO et al., 2015)
			ELISA	27/50 (54)	

			IFAT	25/50 (50)	
			qPCR (msp1α gene)	19/50 (38)	
MS					
MS	Survey	667	Complement fixation	53/667 (8)	(MADRUGA; AYCARDI; PUTT, 1983)
Campo Grande	Survey	50	Card agglutination test	50/50 (100)	(MADRUGA et al., 1985)
Miranda	Survey	128	ELISA	102/128 (79.7)	(SILVA et al., 2006)
			ELISA (rMSP5)	100/128 (78.1)	
MT	Survey	516	Indirect ELISA	212/516 (41.1)	(SILVA et al., 2015b)

Abbreviations: AC, Acre; BA, Bahia; ES, Espírito Santo; GO, Goiás; MG, Minas Gerais; MS, Mato Grosso do Sul; MT, Mato Grosso; PA, Pará; PB, Paraíba; PE, Pernambuco; PI, Piauí; PR, Paraná; RJ, Rio de Janeiro; RO, Rondônia; RS, Rio Grande do Sul; SC, Santa Catarina; SE, Sergipe; SP, São Paulo; TO, Tocantins; ELISA, Enzyme-linked Immunosorbent Assay; IFAT, immunofluorescence antibody test, N/I; not informed.

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4. FIRST REPORT OF *ANAPLASMA MARGINALE* INFECTION IN GOATS, BRAZIL

Running Title: *Anaplasma marginale* in goats

Abstract

We report the first molecular detection of *Anaplasma marginale* in goats from northeastern Brazil, based on *msp*4 and *msp5* gene sequencing analysis. Sequencing of the detected *A. marginale msp1α* gene revealed that goats were infected with genotype F. *Amblyomma parvum* and *Rhipicephalus microplus* were found feeding on animals.

Anaplasma marginale, the causative agent of bovine anaplasmosis, is a tick-borne bacterium that causes significant economic losses for cattle industries (KOCAN et al., 2010). *A. marginale* infects cattle through tick transmission worldwide, but increasingly the bacterium is being detected in other domestic and wild ruminants. At least 20 ixodid tick species have been implicated in the transmission of *A. marginale*, including *Dermacentor* spp. and *Rhipicephalus* spp. (KOCAN et al., 2010).

Anaplasma marginale is found in regions where tick vectors are endemic. In tropical and subtropical regions, *Rhipicephalus microplus* is the vector of bovine anaplasmosis. Despite host specificity of *R. microplus* for cattle (MA et al., 2016), this tick species may be found parasitizing small ruminants (BRITO; SANTOS; GUERRA, 2005). In Brazil, *R. microplus* is endemic and hampers livestock production resulting in annual economic losses estimated at U$ 3.24 billion (GRISI et al., 2014).

In northeastern Brazil, multispecies grazing is a common family subsistence practice on smallholder farms possibly facilitating interspecies transmission of pathogens. A study of co-grazing ruminants has shown a single *A. marginale* strain infecting coexisting cattle, buffalo and ticks (SILVA et al., 2014). *A. marginale* infection has been previously molecularly described in sheep from Iran (YOUSEFI et al., 2017). However, to the best of our knowledge, *A. marginale* has never been detected in goats. This study aimed to estimate the prevalence of *A. marginale* and factors associated with the infection in goats from the State of Paraíba, northeastern Brazil.

4.1. The study

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 403 blood samples from goats (368 females and 35 males), previously surveyed for other pathogens (MACHADO et al., 2017), were included in this study. All samples were collected in an anticoagulant tube containing ethylenediaminetetraacetic acid (EDTA) and stored at -80 °C. An epidemiological questionnaire was given to each farm owner addressing age, gender, presence of ticks and multispecies grazing. The age of goats was stratified into groups of ≤ one year and > one year old.

Genomic DNA was extracted from 200 µL of whole blood using a commercial kit (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. Negative controls using ultra-pure water were performed in parallel to monitor cross-contamination in each batch of 30 samples.

PCR amplification of the caprine 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*- and *A. ovis*-infection using previously described primers targeting the *Anaplasma* spp. *msp4* gene (≈870 bp) (DE LA FUENTE et al., 2007). Amplified DNA fragments of the *msp4* gene from two *Anaplasma* spp. isolates were directly sequenced using the Sanger method, and analyzed sequences compared by BLASTn with those present in the GenBank® database.

The identity of *A. marginale* in the blood was confirmed by PCR amplification of *msp5* followed by sequencing. *Anaplasma* genotypes were differentiated by amplifying, cloning and sequencing of the repeat region of the *msp1α* gene as previously described (CASTAÑEDA-ORTIZ et al., 2015).

Either the Chi-square or the Fisher's exact test was used to assess association of the individual factors such as age, gender, presence of ticks, and multispecies grazing with *Anaplasma* spp. infection. *P*-values were calculated and considered significantly different when $P < 0.05$. Data was compiled and analyzed by Epi Info™ Software (version 7.1.5, CDC).

4.2. Conclusions

This study describes the first molecular report of *A. marginale* in goats. Eleven out of 403 goats (2.73%; CI 95%: 1.53-4.82%) were positive for the *Anaplasma msp4* gene. All analyzed *Anaplasma* spp. *msp4* gene sequences showed ≥99% identity to multiple *A. marginale*

msp4 gene sequences deposited in GenBank (KX989533, AY283196, EU283844, AY702919, CP001079). Moreover, sequencing of the *msp5* gene revealed the presence of *A. marginale* sensu stricto. Anti-*Anaplasma* spp. antibodies have been identified in goats from northeastern Brazil (RAMOS et al., 2008). However, direct molecular detection of *A. marginale* in small ruminants has been only reported in sheep from Iran (YOUSEFI et al., 2017).

The rate of *A. marginale*-infection and corresponding estimated parameters for each of the evaluated potential risk factor is shown in Table 1. Tick-infested goats were six times more likely to be infected with *A. marginale* ($P = 0.02788$). The tick species feeding on the studied goats were identified as *Amblyomma parvum* (49/52, 94.23%) and *R. microplus* (3/52, 5.77%). *R. microplus* has been described as the main vector of *A. marginale* (KOCAN et al., 2010), yet no study has tested the vector competence of *A. parvum* for *A. marginale*. However, previous studies have suggested that *Amblyomma* ticks may be involved in the transmission of *A. marginale* (DA SILVA; DA FONSECA; BARBOSA, 2015; SILVA et al., 2014). Further studies are necessary to evaluate if *A. parvum* is a competence vector of *A. marginale* in Brazil.

All *A. marginale*-positive goats were found on farms with multispecies grazing ($P= 0.04300$). Co-grazing of goats, sheep and cattle is the most common practice of northeastern Brazil, since it allows a wider diversification of products for commercialization. A previous study in Rio de Janeiro State, southeastern Brazil, has shown that *A. marginale* strains identified in water buffaloes were closely related to *A. marginale* strains from cattle as determined by sequencing *msp1α* (SILVA et al., 2014).

The molecular weight of MSP1a varies among isolates of *A. marginale* due to the different number of tandemly repeated 23–31 amino acids that constitute part of the protein (CABEZAS-CRUZ et al., 2013). This molecular weight variation may be used to differentiate and characterize geographic strains (CABEZAS-CRUZ et al., 2013). To determine the genotype of the *A. marginale*, we sequenced *msp1α* and found exclusively the *A. marginale* genotype F in studied infected goats. In Brazil, six microsatellite genotypes have been reported in cattle, namely, B, C, D, E, G and H, with genotype E being the most common (MACHADO et al., 2015; POHL et al., 2013). In this study, goats were infected with F genotype. It is unknown whether this genotype is goat specific *A. marginale*. Additional studies are necessary to elucidate this question.

This study gives the first insight into *A. marginale* infection in goats from Paraíba State, northeastern Brazil and demonstrates that goats may play a role in the epidemiology of

A. marginale as a yet unrecognized reservoir host. Competent ticks feeding on goats and cattle may transfer the pathogen between the two livestock species.

Acknowledgments

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Brief biographical sketch of first author

Nayara Bezerra da Silva is a Master Student in the Graduate College of Veterinary Sciences of the Universidade Federal do Paraná. Her research interests focus on the epidemiology of tick-borne diseases.

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Table 1. Prevalence of *Anaplasma marginale* in goats within each variable studied, Paraíba State, northeastern Brazil.

		A. marginale – msp4				
		+/n	**(%)**	**OR**	**95% CI**	***P*-value**
Age	>1	10/337	2.97	1.9878	0.25-15.79	0.43814
	≤1*	1/66	1.52			
Gender	Female	10/368	2.72	0.9497	0.12-7.64	0.63673
	Male*	1/35	2.86			
Presence of ticks	Yes	3/26	11.54	6.0163	1.49-24.21	0.02788
	No*	8/377	2.12			
Multispecies grazing	Yes	11/304	3.62	†	†	0.04300
	No*	0/99	0.00			

Abbreviations: *msp4*, major surface protein 4; +, Number of positive animals; n, number of samples; 95% CI, 95% confidence interval; †, not applicable; *, reference.

5. FINAL CONSIDERATIONS

In Brazil, *A. marginale* infection has been widely described in cattle, however, little is known about it in other animal species. This is the first molecular report of *A. marginale* infection in goats. Additionally, we describe for the first time the genotype F in Brazil. This study gives the first insight into *A. marginale* infection in goats from Paraíba State, northeastern Brazil and demonstrates that goats may play a role in the epidemiology of this bacterium as a yet unrecognized reservoir. Competent ticks feeding on goats and cattle may transfer the pathogen between the two livestock species.

6. APPENDIX

Appendix 1. Approval from the Ethics Committee

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

NOTIFICAÇÃO

João Pessoa, 25 de setembro de 2014
CEUA No 3305/14

Ilmo(a). **Rafael Felipe da Costa Vieira**

Departamento **Ciências Veterinárias - CCA - UFPB**

Orientando(a): **Rafael Felipe da Costa Vieira**, (**Mestrado**)

A Comissão de Ética no Uso de Animais do Centro de Biotecnologia da Universidade Federal da Paraíba em sua reunião ordinária de **25/09/2014** analisou e **APROVOU** a execução do projeto **Caracterização epidemiológica de patógenos transmitidos por carrapatos, Leishmania sp., Toxoplasma gondii e Neospora caninum em caprinos e equinos do Estado da Paraíba.**

Com previsão de empregar **380 Caprinos, 380 Equídeos - Propriedades rurais**.

Para serem utilizados no período de **01/05/2014** a **31/07/2016**

Atenciosamente,

Prof. Dr. Luis Cezar Rodrigues
Presidente da Comissão de Ética no Uso de Animal do CBiotec/UFPB

Appendix 2. Epidemiological questionnaire

QUESTIONARIO EPIDEMIOLOGICO - CAPRINOS

DADOS PROPRIETARIO

Propriedade | ID Animal | ID Amostra | Data coleta

Proprietario

Cidade | Telefone ###.###.####

ANIMAIS

1. Possui animais em casa?

SANEAMENTO

3. Qual a origem da agua de consumo?

◯ rede publica ◯ poco ◯ rio/corrego ◯ mineral

4. Qual o destino do esgoto?

◯ rede publica ◯ fossa ◯ ceu aberto ◯ rio/corregos

5. Qual o destino do lixo de sua casa?

◯ coleta publica ◯ quintal ◯ queima

◯ rede publica ◯ poco ◯ rio/corrego ◯ mineral

4. Qual o destino do esgoto?

◯ rede publica ◯ fossa ◯ ceu aberto ◯ rio/corregos

5. Qual o destino do lixo de sua casa?

◯ coleta publica ◯ quintal ◯ queima

6. Possui horta em casa?

7. Os vegetais sao higienizados adequadamente?

Yes

FELIDEOS

8. Ha felinos na propriedade?

9. Qual o destino das fezes?

◯ solo ◯ fonte de agua ◯ lixo comum

10. Tem livre acesso a criacao e/ou aos reservatorios de alimentos dos animais?

11. É fornecido carne crua ou mal cozida a esses animais?

12. Tem livre acesso a restos placentarios, fetos abortados ou tecidos fetais?

CANIDEOS

13. Ha presenca de canideos na propriedade?

14. Qual destino das suas fezes?

◯ solo ◯ fontes de agua ◯ lixo comum

15. Tem livre acesso a criacao e/ou aos reservatorios de alimentos dos animais?

16. É fornecido carne crua ou mal cozida a esses animais?

17. Tem livre acesso a restos placentários, fetos abortados ou tecidos fetais?

SISTEMA REPRODUTOR

18. Ha relatos de repeticao de cio?

19. Nascimento de animais fracos, natimortos ou abortos?

20. Fetos mumificados ou macerados?

21. nascimento de animais com problemas articulares e/ou no sistema nervoso?

18. Ha relatos de repetição de cio?

19. Nascimento de animais fracos, natimortos ou abortos?

20. Fetos mumificados ou macerados?

21. nascimento de animais com problemas articulares e/ou no sistema nervoso?

CONTROLE DE CARRAPATOS

22. Controle de carrapatos?

23. Qual produto?

◯ Ivermectina ◯ Doramectina ◯ Colosso ◯ Cipermetrin

24. Uso de carrapaticida?

25. Frequencia controle carrapatos:

◯ semestral ◯ 1x ano ◯ ano todo

26. Rotacao de principio ativo?

27. Presenca de carrapatos?

INFORMACOES INDIVIDUAIS

DADOS

ID Animal

Sexo

◯ Fem ◯ Masc

Idade

Pelagem

Raça

◯ SRD
◯ BOER
◯ SAANEN
◯ PARDA ALPINA

Idade

◯ =<1
◯ >1

Aptidão

◯ Corte
◯ Leite
◯ Exposição

EXAMES

Hematocrito

Proteina Total

Micoplasma

Neospora-RIFI-1:50

Coletado carrapato

Espécie de carrapato

◯ *A. parvum*
◯ *R. (Boophilus) microplus*
◯ *A. parvum + R. (Boophilus) microplus*

Hematócrito

◯ >=22 ◯ <22

Pronteina Total

◯ <6.4 ◯ >7
◯ 6.4-7

Proprietario

1. Idade do criador?

◯ 20-30 ◯ 31-40 ◯ 41-50 ◯ 51-60 ◯ 61-70 ◯ 71-80

2. Estado civil?

◯ Solteiro ◯ Casado ◯ Separado ◯ Viuvo ◯ Concubinato

3. Escolaridade

◯ Analfabeto ◯ EF Completo ◯ EM Completo ◯ ES Incompleto
◯ EF Incompleto ◯ EM Incompleto ◯ ES Completo

4. Voce ou outras pessoas da familia ou trabalhadores ja sofreram alguma doenca relacionada a criacao de animais?

5. Tamanho do rebanho

◯ 2-10 ◯ 11-20 ◯ 21-30 ◯ 31-40 ◯ 41-50 ◯ Acima 50

6. Sistema de criacao?

◯ Extensivo ◯ Intensivo ◯ Semi intensivo

7. Fonte de agua

◯ Parada ◯ Corrente ◯ Parada e corrente

8. Qual o tipo de alimentacao?

6. Sistema de criacao?

◯ Extensivo ◯ Intensivo ◯ Semi intensivo

7. Fonte de agua

◯ Parada ◯ Corrente ◯ Parada e corrente

8. Qual o tipo de alimentacao?

◯ Capim ◯ Racao ◯ Outra ◯ Capim racao e palma
◯ Feno ◯ Palma ◯ Capim e racao ◯ Capim e outro

9. Rotacao de pastagem?

10. Animais recebem mineralizacao?

11. Criacao consorciada?

12. Disturbios reprodutivos nos animais?

13. Utiliza animais de outra propriedade pra reproducao?

14. Crias recebem colostro? No

15. Tratamento termico no colostro?

16. Tem caes ou gatos na propriedade?

17. Alimentacao desses animais?

◯ Racao ◯ Visceras de animais abatidos na propriedade
◯ Comida ◯ alimento caseiro
◯ Sobras de comida ◯ leite e farelo de milho

Ativar o Windows
Acesse Configurações para a

VITA

Nayara Bezerra da Silva is a Master Student in the Graduate Program in Veterinary Sciences at the Universidade Federal do Paraná. Her research interests focus on the epidemiology of tick-borne diseases.