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Original Article
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Genetic diversity of *Anaplasma marginale* in calves under natural transmission conditions in the Northeast region of Pará[1]

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ABSTRACT.- Monteiro M.V., Lima D.H.S., Barbosa J.D., Coelho K.F., Cordeiro M.D., Fonseca A.H., Magalhães-Matos P.C. & Silveira N.S.S. 2023. **Genetic diversity of *Anaplasma marginale* in calves under natural transmission conditions in the Northeast region of Pará**. *Pesquisa Veterinária Brasileira 43:e07158, 2023.* Instituto de Medicina Veterinária, Campus Castanhal, Universidade Federal do Pará, Rodovia BR-316 Km 61, Castanhal, PA 68741-740, Brazil. E-mail: nataliasilva@ufpa.br

The objective of the present study was to detect the genetic diversity of *Anaplasma marginale* strains in naturally infected calves from a rural property located in the northeastern region of the state of Pará, Eastern Amazon, which has a history of mortality due to anaplasmosis. Fourteen calves positive for *A. marginale* were selected using a semi-nested polymerase chain reaction for the target msp1α gene, with asymptomatic (n=3) and symptomatic (n=11) infections. After sequencing the samples, two genotypes were verified in the E and C regions and the structures in tandem repeats were determined. Nine different strains were found: eight related to the E genotype (α-β-β-Γ = one animal, asymptomatic; 16-F-17-F-F = two animals, symptomatic; α-β-F-F-F-F = one animal, asymptomatic; 31-62-62-61 = one animal, symptomatic; τ-10-3 = three animals, two symptomatic and one asymptomatic; α-β-β-β = one animal, symptomatic; τ-22 -13-18 = two animals, both symptomatic; β-β-β-BRA1-31 = two animals, both symptomatic), and one related to genotype C (23-24-25-31-27-27 = one animal, asymptomatic). Genotype E was predominant in 92.86% of the samples (13/14), followed by genotype C (7.14%). This study made it possible to detect the genetic diversity of *A. marginale* in calves from the selected dairy farm, in addition to identifying the BRA1 sequence in the animals of the present study, which was recently diagnosed in Minas Gerais, demonstrating the dispersion of *A. marginale* strains in herds from different Brazilian states. Genetic diversity of *A. marginale* was observed in both symptomatic and asymptomatic calves. There were no significant differences when clinical signs were compared to the genotype verified in the infected animals. The prevalence of pathogenicity was not observed.

INDEX TERMS: Semi-nested PCR, sequencing, *Anaplasma marginale*, *msp1α*, BRA1, calves.

RESUMO.- [**Diversidade genética de *Anaplasma marginale* em bezerros sob condições de transmissão natural na região Nordeste do Pará.**] O objetivo do presente trabalho foi detectar a diversidade genética de cepas de *Anaplasma marginale* em bezerros naturalmente infectados oriundos de uma propriedade rural localizada na região nordeste do estado do Pará, Amazônia Oriental, a qual apresentava histórico de mortalidade devido à anaplasmose. Foram selecionados 14 bezerros positivos para *A. marginale* pela técnica de semi-nested PCR (nPCR) para o alvo no gene *msp1α*,

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com infecção assintomática (n=3) e sintomáticos (n=11). Após o sequenciamento das amostras foram verificados dois genótipos nas regiões E e C, e determinadas as estruturas em tandem *repeats.* Nove diferentes estirpes foram encontradas, sendo oito relacionadas ao genótipo E (α-β-β-Γ = um animal, assintomático; 16-F-17-F-F = dois animais, sintomáticos; α-β-F-F-F-F = um animal, assintomático; 31-62-62-61 = um animal, sintomático; τ-10-3 = três animais, dois sintomáticos e um assintomático; α-β-β-β = um animal, sintomático; τ-22-13-18 = dois animais, sintomáticos; β-β-β-BRA1-31 = dois animais, sintomáticos) e uma relacionada ao genótipo C (23-24-25-31-27-27 = um animal, assintomático). O genótipo E foi predominante em 92,86% das amostras (13/14), seguido pelo genótipo C (7,14%). O estudo possibilitou a detecção da diversidade genética de *A. marginale* em bezerros dessa propriedade leiteira, além de identificar a sequência BRA1 nos animais do presente estudo, a qual foi diagnosticada recentemente em Minas Gerais, o que demonstra a dispersão das estirpes de *A. marginale* nos rebanhos de diferentes estados brasileiros. A diversidade genética de *A. marginale* foi observada tanto em bezerros sintomáticos quanto em assintomáticos e não houve diferença significativa quando se comparou os sinais clínicos ao genótipo verificado no animal infectado, não observando a prevalência de patogenicidade de estirpes.

TERMOS DE INDEXAÇÃO: Semi-nested PCR, sequenciamento, *Anaplasma marginale*, *msp1α*, BRA1, bezerros.

INTRODUCTION

Anaplasma marginale (Theiler, 1910) is an intraerythrocytic bacterium that can be transmitted biologically by *Riphicephalus microplus* ticks, mechanically by blood-sucking flies, infected blood on fomites, and transplacentally (Kocan et al. 2004, 2010, Aubry & Geale 2011). This pathogen causes bovine anaplasmosis, a disease of great economic importance in South America (Grau et al. 2013, Silva et al. 2021).

Clinical signs of bovine anaplasmosis include fever, anemia, weight loss, lethargy, jaundice, gastrointestinal disturbances, decreased milk production, abortion, and death (Kocan et al. 2003, 2010). Brazil is classified as an area of endemic stability for bovine anaplasmosis owing to the constant transmission of the agent. Thus, to understand the epidemiological situation of herds, in addition to serological analysis and detection (Silva et al. 2015a), molecular characterization of the different strains of *A. marginale* circulating in the national territory is necessary (Silva et al. 2015). The molecular data are obtained by polymerase chain reaction (PCR); examples include studies of the genetic diversity of *A. marginale* by De la Fuente et al. (2004) and Bahia et al. (2021).

Genetic markers of lineages and phylogenetic studies of *A. marginale* are the genes that encode major surface proteins (MSPs), which are involved in the interactions between host and pathogen, and pathogens and ticks (De la Fuente et al. 2007). To date, six MSPs have been identified in *A. marginale* derived from bovine erythrocytes, conserved in tick-derived organisms and cell cultures. They stimulate the production of antibodies in animals naturally infected or vaccinated with early bodies of *A. marginale* (Tebele et al. 1991). MSP1a, MSP4, and MSP5 are encoded by a single gene, whereas MSP1b, MSP2, and MSP3 belong to multigenic families and can vary antigenically in persistently infected cattle (Cabezas-Cruz et al. 2013).

MSP1a is encoded by a single gene, *msp1α* (Allred et al. 1990). It is used as a stable genetic marker for identifying geographic isolates worldwide (De la Fuente et al. 2007, Cabezas-Cruz et al. 2013). Repeated sequences of MSP1a surface proteins have been demonstrated in cattle in the states of Rio de Janeiro (Silva et al. 2015), São Paulo (Silva et al. 2016), and Minas Gerais (Pohl et al. 2013, Bahia et al. 2021). In Pará, studies identifying the genetic variation of A. marginale strains in this region have not yet been reported. Therefore, the objective of the present study was to detect the genetic diversity of *A. marginale* strains in naturally infected calves from a rural property located in the northeastern region of the state of Pará, Eastern Amazon, which has a history of mortality due to anaplasmosis.

MATERIALS AND METHODS

Ethical aspects. This study was approved by the Ethics Committee for the Use of Animals of the Federal University of Pará (CEUA/UFPA) according to protocol number 5264261020.

Study area. The study was carried out on a dairy farm located 21km from the city of Castanhal (1°07'19.1" S and 47°53'53.0" W), in the northeast region of the state of Pará, with a history of recurrent clinical cases and death of animals by bovine anaplasmosis, even with early treatment (Barbosa 2018).

Animals. The selection of calves was conditioned by the availability of the farm on which births occurred from April to May 2021 due to the fixed-time artificial insemination protocol established on the property. Of 24 crossbred Gir and Holstein calves monitored weekly from the first day of birth to 60 days of age, 14 were selected for the study of genetic diversity by nested polymerase chain reaction (nPCR).

The calves were divided into three age groups for better monitoring: 1-20, 21-41, and 42-60 days. The calves were born in a maternity paddock. They ingested colostrum for the first six hours of life and stayed with their mother for up to 20 days. They were then kept collectively with animals of other age groups with ad libitum access to mineral salt, feed, and water. During this period, they were released to the pasture in the morning and spent the afternoon with their mothers.

Collection and processing of samples. The first blood collection occurred 24 hours after birth, after 20 days, and then once a week until two months of age. A total of 192 blood collections were performed from the 24 experimental animals. Blood samples were collected by jugular venipuncture in sterile vacuum tubes containing the anticoagulant ethylenediaminetetraacetic acid (K3EDTA). Aliquots were stored in Eppendorf tubes in a freezer (-20°C) for subsequent DNA extraction and PCR.

snPCR for *msp1α* gene. The *msp5* positive samples were evaluated for the presence of *msp1α* target genes by semi-nPCR using oligonucleotide primer sequences 1733F (5'-TGTGCTTATGGCAGACATTTCC-3'), 3134R (5'-TCACGGTCAAAAACCTTTTGCTTACC-3'), and 2957R (5'-AAACCTTGTAGCCCCAACTTATCC-3') (Lew et al. 2002). Amplification was performed under the following conditions: initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 30 s, 55°C for 60 s (for the first reaction) and 60°C for 60 s (for the second reaction), 72°C for 2 min, with a final extension at 72°C for 7 min. The first and second reactions were performed in a final volume of 25µl with the reagents at the concentrations described in the nPCR above. The nPCR products were analyzed by 1.5% agarose gel electrophoresis and stained with ethidium bromide.

DNA sequence analysis for *msp1α* gene. The semi-nPCR amplified products were purified. Sequencing of the products amplified in both directions was performed using an automated method based on chain termination by dideoxynucleotides, using a microsatellite located in the 5'- untranslated region (UTR) of the *msp1α* gene between the putative Shine-Dalgarno sequence (GTAGG) and the translation initiation codon (ATG), whose structure is GTAGG (G/ATTT) m (GT) n T ATG, with the SD-ATG distance calculated using the formula (4 × m) + (2 × n) + 1 (De la Fuente et al. 2001, Estrada-Peña et al. 2009, Silva et al. 2015). Sequencing was performed at "Universidade Estadual Paulista 'Júlio de Mesquita Filho'" (Unesp), Jaboticabal, São Paulo.

Statistical analysis. Fisher's exact test was used to verify whether the genotype was associated with clinical signs in the studied animals. Analyses were performed using Bioestat 5.3 program, adopting a significance level of α=0.05.

RESULTS

Samples from 14 calves positive for the *msp1α* gene of *Anaplasma marginale* (Theiler, 1910) were sequenced. Three animals were asymptomatic, and 11 were symptomatic, with a rate of change in packed cell volume from 10 to 33%. From the selected samples, nine strains responsible for infection in the studied calves were found: τ-22-13-18 (two animals), 16-F-17-F-F (two animals); α-β-β-β-BRA1-31 (two animals), τ-10-3 (three animals), α-β-β-β (one animal), α-β-F-F-F-F (one animal), 31-62-62-61 (one animal), α-β-β-β-Γ (one animal), and 23-24-25-31-27-27 (one animal).

In the analysis of *msp1α* from *A. marginale*, the presence of the E and C genotypes was observed, with a predominance of the E genotype, which has a Shine-Dalgarno (SD) sequence and an ATG translation initiation codon (SD-ATG) at a distance of 23 nucleotides. Animals with more than one genotype were not observed. In contrast, genotype C has SD-ATG distances of 19 nucleotides, presenting a non-symptomatic animal, representing 7.14% of the total sequenced, with a hematocrit rate of 10% (Table 1). Of the 14 samples sequenced in the analysis of the tandem repetitions of *msp1α*, the E genotype was found in 92.86% of the animals. Three of these animals were asymptomatic, and 10 were symptomatic (fever, pale mucous membranes, apathy, enlarged lymph nodes, increased heart rate, and diarrheal stools). The lowest hematocrit (13%) occurred at 54 days of age. No clinical deaths occurred.

The proportion of animals infected with genotypes E and C with specific clinical signs did not differ significantly (p=0.286).

From the sequenced tandem repetitions, eight strains of the E genotype were identified. Strain 16-F-17-F-F was present in two animals at 26 and 41 days of age; strain τ-10-3 in three animals at 34, 47, and 54 days of age, strain τ-22-13-18 in two animals at 54 days of age; and strain α-β-β-β-BRA1-31 found in two 54-day-old animals. Strain 23-24-25-31-27-27 was identified with genotype C.

DISCUSSION

The higher frequency of clinical anaplasmosis cases may be associated with the wide genetic diversity of *Anaplasma marginale*. Studies have reported different pathogenic strains involved in disease outbreaks in cattle herds in the Americas (Alamzán et al. 2008, Ruybal et al. 2009, Machado et al. 2015, Silva et al. 2015, Bahia et al. 2021). In the present study, the E and C genotypes of *A. marginale* were identified, with a prevalence of the former. The finding indicates that the E genotype is better adapted with SD-ATG distances of 23 nucleotides and variation from two to seven copies of tandem repeats. According to Estrada-Peña et al. (2009), microsatellite genotypes with high levels of tandem repeats of MSP1 protein expression are more efficient in infecting reservoir hosts. Both genotypes have been reported in cattle in Brazil, specifically in Rio de Janeiro (Silva et al. 2015) and Minas Gerais (Bahia et al. 2021).

The transplacental transmission of *A. marginale* has been described by Grau et al. (2013) and Henker et al. (2020). Kocan et al. (2010) reported that pregnant cows with rickettsemia and acute clinical signs might abort. Although the molecular evaluation of the cows was not the objective of the present study, there were no clinical signs of anaplasmosis in the peripartum or postpartum period. Calves up to 24 h after birth were negative in nPCR molecular analysis for *A. marginale*. Thus, due to the increase in infected animals that occurred from 20 to 60 days, it is believed that there was no

Table 1. Identification of *Anaplasma marginale* isolates found in calves on a dairy farm, Northeastern Pará, Eastern Amazon, Brazil

Age (days)	Genotype	Tandem repeat structure of *msp1α*	SD-ATG distance	m*	n**	Presence of clinical sign	Ht %[1]
20 days	E	α-β-β-Γ	23	2	7	No	33
26 days	E	16-F-17-F-F	23	2	7	Yes	28
34 days	E	α-β-F-F-F-F	23	2	7	No	23
34 days	E	31-62-62-61	23	2	7	Yes	20
34 days	E	τ-10-3	23	2	7	No	24
41 days	C	23-24-25-31-27-27	19	2	5	Yes	10
41 days	E	α-β-β-β	23	2	5	Yes	35
41 days	E	16-F-17-F-F	23	2	7	Yes	26
41 days	E	τ-10-3	23	2	7	Yes	24
54 days	E	τ-22-13-18	23	2	7	Yes	19
54 days	E	τ-22-13-18	23	2	7	Yes	13
54 days	E	α-β-β-β-BRA1-31	23	2	7	Yes	13
54 days	E	τ-10-3	23	2	7	Yes	19
54 days	E	α-β-β-β-BRA1-31	23	2	7	Yes	22

* Number of repeats of the G/ATTT nucleotide sequence, ** number of repeats of the G nucleotide sequence; [1] hematocrit (blood volume).

transplacental infection in the animals studied. According to De Andrade (2004), this age group is within the incubation period of *A. marginale* and within a possible immunological window for the parasite. In the present study, once infected, animals remained infected until the end of the experiment. These findings corroborate those reported by Lima et al. (2019), who experimentally observed the persistence of *A. marginale* infection in buffalo and bovine calves.

It is believed that the main epidemiological factors that contributed to the maintenance of the agent on the property are the constant presence of *Rhipicephalus microplus*, associated with the acquisition of animals from other properties and possible carriers of the agent. Kocan et al. (2010) and Bahia et al. (2021) reported that these factors associated with the inappropriate use of animal markings and/or vaccination may contribute to the maintenance of *A. marginale* in herds. In the age group studied, there was no practice of vaccination or worming performed during the period with one needle per animal.

The molecular weight of protein MSP1α differs among different strains of *A. marginale*. This occurs because of a variable number of tandem repeats of 19 to 31 amino acids, both in the amino- and N-terminal regions of the protein. The number of repeats varies among geographic isolates of *A. marginale* and is a stable marker of strain identity (Estrada-Peña et al. 2009, Pohl et al. 2013).

In the present study, there was a predominance of the E genotype, which has a SD-ATG at distances of 23 nucleotides, correlated to a microsatellite with a high expression level of the MSP1α protein (Estrada-Peña et al. 2009). This suggests a high potential for infectivity of the identified sequences, indicating that the E genotype is better adapted to the herd and that it is the genotype that presently displayed good transmission capacity in relation to the C genotype.

Estrada-Peña et al. (2009) evaluated nine different genotypes in four ecosystems worldwide, and in South America, especially Brazil and Argentina. Genotype E was the most common type, similar to the present observations. In addition, genotypes B, C, D and G were previously detected in Argentina and Brazil (De la Fuente et al. 2004, Vidotto et al. 2006, Ruybal et al. 2009, Pohl et al. 2013, Silva et al. 2015). The present study detected genotype C in Pará.

Genetic diversity can be explained by the constant movement of cattle in the region, host-parasite interactions and the consequent evolutionary pressure. The property studied has a history of buying cows from different municipalities in the state of Pará, and some from other states. Palmer et al. (2004) identified 11 strains of cattle at the State University of Kansas, and in five animals there identified two strains of *A. marginale* with different genotypes, indicating superinfection, different from what was found in the present study, in which no animal presented infection by more than one genotype.

A similarity of 99.08% of the α-β-F-F-F-F strain with the α-β-F-F-F strain was reported in a study carried out in Minas Gerais (Bahia et al. 2021). The authors associated the strain with acute clinical cases of anaplasmosis. Therefore, they suggested that the α-β-F-F-F strain caused disease in calves on the studied farms in that state. On the other hand, in the present study carried out in Pará, the α-β-F-F-F-F strain was found in asymptomatic animals. It had four copies of tandem repeats, and one more sequence for the F structure, indicating that the degree of pathogenicity is not high. However, it is a factor of enzootic stability by maintaining the presence of carrier animals in the herd, which may be associated with the geographic location and climatic factors favorable to the biology of the vector. Furthermore, calves from areas of enzootic stability for hemoparasites rarely develop clinical diseases (Solorio-Rivera et al. 1999). Despite this, Carvalho et al. (2012) drew attention to the fact that calves, even in areas of enzootic stability, may present with cases of clinical anaplasmosis, similar to the present study.

The tandem repeat sequences of *msp1α* (α-β-β-Γ and α-β-β-β) obtained in the present study were genetically identical to those obtained by Vidotto et al. (2006) in the state of Paraná. The researchers reported high similarity (100%) with the strains from Mexico (α-β-β-Γ) and Minas Gerais, Brazil (90-97%) (Vidotto et al. 2006, Pohl et al. 2013). Ramos et al. (2019) evaluated the genetic diversity of *A. marginale* using the *msp1α* gene in the Brazilian Pantanal and found the genetic presence of this strain in the region. These studies demonstrate the dispersion of the strain, which can cause mild to severe clinical cases, according to each region studied. In the present study, the α-β-β-Γ strain did not trigger clinical signs, unlike the α-β-β-β strain, which triggered clinical symptoms but did not cause animal death.

According to Cabezas-Cruz et al. (2013), the strain τ-22-13-18 is commonly found in Argentina and Mexico. This strain was also identified in the present study and was associated with a decrease in hematocrit (13-19%) and clinical signs, such as apathy, hyperthermia, dehydration, pale mucous membranes, and diarrhea.

CONCLUSIONS

The genetic diversity of *Anaplasma marginale* is revealed in dairy calves from a property with a history of deaths by *A. marginale*, located in the northeast of Pará, Eastern Amazon, Brazil. Eight strains were observed (τ-22-13-18, 16-F-17-F-F, α-β-β-β-BRA1-31, τ-10-3, α-β-β-β, α-β-F-F-F-F, 31-62-62-61, and α-β-β-Γ). The E genotype was predominant.

The identification of the BRA1 sequence in animals from the present study, which was recently diagnosed in animals from Minas Gerais, demonstrates the rapid dispersion of *A. marginale* strains in herds from different states and highlights the vulnerability of cattle to the agent.

The genetic diversity of *A. marginale* was observed in symptomatic and asymptomatic calves. There was no significant difference between the clinical signs and the genotype verified in infected animals. Therefore, no pathogenicity of the strains was observed.

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Conflict of interest statement.- The authors declare that they have no conflict of interest.

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