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Short Communication

Molecular characterization of the *msp1 α* AmRio1 strain of *Anaplasma marginale* in calves and experimentally infected ticks

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ABSTRACT

This study aims to evaluate the infection's clinical parameters and genetic diversity of *msp1α* of the AmRio1 strain in acute and chronic infections in cattle and ticks. A calf experimentally infected with the *A. marginale* AmRio1 strain was monitored during acute infection, and the presence of the *msp1α* gene was verified in the semi-nested polymerase chain reaction (PCR) during the acute and persistent phases of infection. From day seven after inoculation of the pathogen, the calf showed an increase in body temperature, decrease in hematocrit and increase in the percentage of cells infected by the agent, as well as clinical signs. Blood samples from the experimentally infected calf were positive during the acute infection and the persistent PCR infection for the *msp1α* gene. During the acute phase, infestation with *Rhipicephalus microplus* was performed. To evaluate the chronic phase, a blood sample was collected at 90 days post-infection. There was no variation of the MSP1a protein in this study. The AmRio1 strain was pathogenic as it caused severe changes in the clinical parameters of the monitored cattle. The positivity of this strain in organs and saliva of the analyzed ticks indicates a probable biological transmission.

1. Introduction

Bovine anaplasmosis, a disease caused by the intra-erythrocytic rickettsia *Anaplasma marginale* (Rickettsiales: Anaplasmataceae), is economically significant in cattle in tropical and subtropical regions of the world (Kocan et al., 2010; Kocan et al., 2004).

The symptoms of clinical disease are fever, anemia, icterus, weight loss, abortion, and lethargy. Severity and death rate increase with advancing age (Kocan et al., 2010)

Six major surface proteins (MSPs) were described in *A. marginale* infecting bovine erythrocytes and cells in cell culture (Kocan et al., 2000). Three of these (MSP1a, MSP4 and MSP5) are encoded by a single gene, and do not vary their expression during multiplication of the bacterium, while another three (MSP1b, MSP2 and MSP3) are from multigenic families and may vary antigenically owing to the combination of genes that is expressed (Kocan et al., 2003).

The MSP1 protein is a heterodimer formed by two structures, MSP1a and MSP1b, which are unrelated and noncovalently bound (Barbet and Allred, 1991). MSP1a is an adhesin of vertebrate and invertebrate host cells and has a crucial role in *A. marginale* infection (de la Fuente et al., 2001a; de la Fuente et al., 2003; McGarey et al., 1994). This protein has a conserved C-terminal region and variable N-terminal region, where repeated sequences occur, following a stable pattern according to the *A. marginale* strain (de la Fuente et al., 2001a). The sequences may vary in both molecular weight owing to the number of replicates and amino acid alterations present in the replicates (Allred et al., 1990; de la Fuente et al., 2005; de la Fuente et al., 2007). Thus, the *msp1α* gene is considered a stable genetic marker for the identification of strains of *A. marginale* (Bowie et al., 2002; Palmer et al., 2001).

Studies on the diversity of the *msp1α* gene found that further repetitions had a genetic similarity to previously existing sequences, suggesting an evolutionary relationship of the existing sequences in the herd (de la Fuente et al., 2007; Palmer et al., 2001; Silva et al., 2015). These findings are considered evidence that *A. marginale* might be able to coevolve simultaneously with the tick vector (de la Fuente et al., 2007). The alterations found in the amino acid sequences of the MSP1a protein are based on deletions, insertions and amino acid substitutions

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of the repeated sequences (de la Fuente et al., 2007).

Genetic diversity of *A. marginale* was evaluated in *Dermacentor andersoni* ticks (Palmer et al., 2001), but was not in *Rhipicephalus microplus* ticks, that are monoxene and have other feeding dynamic.

Thus, the objective of this study was to evaluate the genetic diversity of the *msp1α* gene of the AmRio1 strain (Baeta et al., 2015) in acute and chronic infection in an experimentally infected immunocompetent vertebrate host and invertebrate host.

2. Material and methods

2.1. Experimental animal

A 20-day-old nonsplenectomized Holstein calf (*Bos taurus*), free of ticks, considered negative for *A. marginale* after conducting the polymerase chain reaction (PCR) for the *msp5* gene (Singh et al., 2012) and observation on optical microscopy slide (Olympus BX41®, Tokyo, Japan) was used for the study. The animal was provided by the dairy cattle sector of UFRRJ and was housed in a screened stall previously treated for removal of ticks.

2.2. Experimental infection of the bovine with the strain, AmRio1, of A. marginale

The AmRio1 strain was propagated in embryonic cells of *Ixodes scapularis* (IDE8 line) as described in Baeta et al. (2015). Culture flasks containing more than 70% infected cells in smears were detached from the remaining IDE8 cells in the vial wall using a sterile Pasteur pipette by rinsing with culture medium contained in the vial. After detachment, the cells were disrupted employing a syringe with a curved needle, by passage under pressure of the medium containing the infected cells. A volume of 1.5 mL of the cell suspension containing *A. marginale* and ruptured IDE8 cells were used for the experimental infection by intravenous inoculation.

2.3. Infestation of the animal

The calf was infested with 1 g (g) of *R. microplus* larvae from a colony of the Porto Alegre strain. This strain was maintained on Holstein calves and kept according to the protocol developed by Reck et al. (2009). The calf was infested with ticks 15 days after ecdysis, this being the 8th day after inoculation of the agent.

2.4. Monitoring of A. marginale infection in cattle

Blood was collected from the stabled calf on days 1, 2, 3, 4, 7, 8, 9, 10, 11, 12, 13, 14, 17, 21, 28 and 90 after experiment infection. Hematocrit, blood smear and rectal temperature measurements of the calf and PCR were performed on the days of collection from 1 to 28 (acute infection). The sample collected at day 90 (persistent infection) was submitted to PCR.

The blood smears were fixed in methanol for 3 min and stained in 10% Giemsa (Sigma-Aldrich®, Darmstadt, Germany) for 45 min and visualized under an optical microscope (Olympus BX41®, Tokyo, Japan) with a 100× immersion objective. The parasitemia was determined according to Ribeiro and Reis (1981). The animal was clinically followed for the observation of possible clinical manifestations, such as mucous pallor, jaundice and prostration.

2.5. Obtaining tick samples

Sixty-one *R. microplus* ticks were recovered 22 days after infestation. Of these, 33 ticks were in the weight range between 100 and 200 mg and 28 ticks in the range of 201 and 300 mg. These ticks were submitted to dissection by the depletion method, through the section of the gnathosoma region using ophthalmic scissors and depletion of idiosoma content by pressure according to Sá et al. (2018). Dissection was performed on Petri dishes containing Phosphate Buffer Saline (PBS) (pH = 7.4)in sufficient volume to cover the tick. The guts and salivary glands were removed and packed into polypropylene tubes containing RNA later in sufficient volume to cover the organ pools. These ticks were grouped into pools of on average of five ticks, resulting in four pools of ticks in the weight range from 100 to 200 mg and four pools between 201 and 300 mg for dissection and harvesting organs. Three pools were intended for collection of saliva, two pools with a weight range from 100 to 200 mg containing six ticks on average, and only one pool between 201 and 300 mg containing five ticks.

Seventeen ticks in total were submitted to the collection of saliva. Pilocarpine (Sigma-Aldrich®, Darmstadt, Germany) was prepared in a concentration of 2% diluted in PBS (pH = 7.4) (Franco et al., 2016), under one of the legs of the last pair of legs to obtain saliva for up to 2 h after the application of the solution.

These ticks were arranged in double-sided tapes, and after application of pilocarpine, their saliva was collected using microhematocrit tubes and packed in polypropylene tubes in pools. During collection of all samples, the tubes remained immersed in ice within styrofoam, and transferred to a freezer at −20 °C by the end of the procedure.

2.6. Molecular analysis of samples

All bovine blood samples and tick samples were extracted using the Qiagen Blood and Tissue kit (Qiagen, Madison, WI, USA). Blood samples of a 200-microliter volume were processed according to the manufacturer's recommendations. Tick organ samples were washed four times by centrifugation in PBS Tween 20 (pH = 7.4) at 16,000*g* for the complete removal of RNA later. The samples were inserted into ATL buffer in the Qiagen Blood and Tissue kit (Qiagen, Madison, WI, USA), with the volume indicated by the manufacturer. The tick organ samples were processed as indicated by the tissue extraction kit. The saliva samples, when they did not reach the final volume of 200 μl, were filled to this volume with PBS Tween (pH = 7.4) to be subsequently subjected to DNA extraction using the same protocol employed for blood, with the Qiagen Blood and Tissue kit (Qiagen, Madison, WI, USA) according to the manufacturer's recommendations.

2.7. Polymerase chain reaction (PCR)

A semi-nested reaction was performed according to the protocol proposed by Lew et al. (2002), which had *msp1α* as the target gene. The following primers were utilized:

1733F: 5′TGTGCTTATGGCAGACATTTCC3′;
3134R: 5′TCACGGTCAAAACCTTTGCTTACC3′;
2957R: 5′AAACCTTGTAGCCCCAACTTATCC3′.

PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide. Subsequently, the positive samples were subjected to purification using the Purelink® PCR Purification kit (Thermo Fisher Scientific), and the purified product was sent for sequencing in both directions.

2.8. Analysis of the diversity of msp1α

The sequences obtained were aligned using the program, CAP 3 (http://doua.prabi.fr/software/cap3), and translated with the program, ExPASy (http://www.expasy.org/).

For the analysis of the sequences, a 5′ UTR microsatellite of the *msp1α* gene, located between the Shine–Dalgarno sequence (GTAGG) and the initiation codon (ATG) sequence, was identified (de la Fuente et al., 2001a). The distance between the Shine–Dalgarno sequence and the codon ATG was calculated using the formula $(4 \times m) + (2 \times n) + 1$ (Estrada-Pena et al., 2009). The repeated sequences were classified according to the nomenclature proposed by de la Fuente et al. (2007).

Table 1
Temperature, number of parasites per field in smears and calf hematocrit in days after inoculation of the strain of *Anaplasma marginale* AmRio1.

Day	Temperature	Blood Smears	Hematocrit
1	39 °C	0,037%	–
4	38,9 °C	0,04%	–
7	39,4 °C	1,6%	–
8	40,2 °C	3,7%	19
9 (parasitemia peak)	40,5 °C	19%	19
10	40,7 °C	–	16
11	40,9 °C	17%	15
12 (transfusion)	40,7 °C	0,37%	14
13 (transfusion)	40,3 °C	–	16
14	39,3 °C	–	16
15	39,0 °C	–	–
17	39,0 °C	–	20
21	38,9 °C	–	22
28	38,5 °C	0,04%	24

Trace-containing parameters (–) were not evaluated.

2.9. Ethical consideration

These procedures were approved by the Ethics Committee on Research of the Federal Rural University of Rio de Janeiro, process number 2134171215.

3. Results

After inoculation of the *A. marginale* AmRio1 strain in the calf, there was an increase in red cell infection accompanied by an elevation of the rectal temperature, reaching hyperthermia at day seven whereas the hematocrit declined (Table 1).

The ninth day after inoculation was considered the peak of parasitemia, with 19% of infected erythrocytes and presentation of clinical signs, such as pale mucous membranes and prostration. The clinical picture of the calf continued to worsen, and poor prognosis was considered on day 12 after inoculation when the hematocrit reached a value of 14%.

Blood samples after inoculation, in the acute phase (days 1 to 28) and in the chronic phase of infection (day 90), and the organs and saliva of all ticks in all ranges were positive for *A. marginale*, with the *msp1α* gene as the target gene (Fig. 1).

The results in the persistent phase of infection (day 90 after infection) of the blood of the experimentally infected animal were also positive for the *msp1α* gene of *A. marginale*.

All samples sequenced after translation showed the replicates of polypeptides belonging to MSP1a of the AmRio1 strain (Table 2).

4. Discussion

Infection with *A. marginale* strain AmRio1 caused changes in clinical parameters, revealing a pathogenic strain. Kocan et al. (2010) affirm that the prepatent period may range from 7 to 60 days and the degree of protection may be influenced by inoculum size, strain virulence, and immunity degree against the disease (Losos, 1986) and (Woldehiwet and Ristic, 1993). The first inclusion of the AmRio 1 strain occurred on day 1 after the inoculum, reaching a peak of parasitemia on the ninth day.

In previous studies, it has been observed that in endemic areas for anaplasmosis, the repeated sequences may exhibit high variability, and more than one repeated sequence of *msp1α* may be found in certain animals (de la Fuente et al., 2001a; Palmer et al., 2001; Silva et al., 2015).

In these endemic areas, the high diversity of *msp1α* has been associated with different transmission events, introducing distinct genotypes maintained subsequently by mechanical or biological transmission (Palmer et al., 2001) besides the contribution of a high population of vectors.

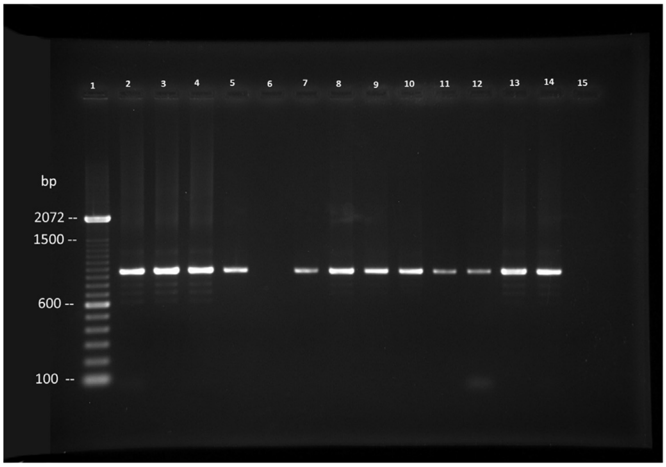

Fig. 1. Photograph of agarose gel 1.5% stained with ethidium bromide. The PCR products come from a semi-nested reaction, according to Lew et al. (2002), which targets the *msp1α* gene. 1- Ladder 100 bp; 2- positive control (DNA of the AmRio1 strain of *A. marginale* in cell culture); 3- Blood of the calf on the day of the parasitemia peak (day 9 after inoculation); 4- Calf blood days after parasitemia peak (day 13 after inoculation); 5- Blood of the calf 3 months after the peak of parasitemia (chronic infection); 6- Negative control (sterile ultrapure water); 7- Pool of tick intestines; 8- Pool of salivary glands of ticks; 9- Pool of salivary glands of ticks; 10- Pool of tick saliva; 11- Pool of tick intestines; 12- Pool of tick intestines; 13- Pool of salivary glands of ticks; 14- Pool of salivary glands of ticks; 15- Negative control (sterile ultrapure water).

Table 2
Amino acids of the MSP1a repeat sequences of the AmRio1 strain of *Anaplasma marginale*, according to de la Fuente et al. (2007).

Sequence of aminoacids	Classification
ADSSSASGQQQES**G**VSSQSGQASTSSQLG	162
TDSSSASGQQQES**S**VSSQSGQASTSSQLG	F
TDSSSASGQQQES**G**VSSQSGQASTSSQLG	17
TDSSSASGQQQES**S**VSSQSGQASTSSQLG	F
TDSSSASGQQQES**S**VSSQSGQASTSSQLG	F

Therefore, sites with a low population of vectors would tend to feature low genetic diversity of *A. marginale* (Ruybal et al., 2009; Silva et al., 2015). Silva et al. (2015) concluded that genetic diversity might be the result of biological, mechanical and transplacental transmission processes taking place simultaneously in a herd, corroborating with Palmer's observations (Palmer et al. (2001).

The present study was conducted for one year, evaluating a bovine during the acute and persistent phase of *A. marginale* infection and its vector ticks. Even so, throughout the evaluation period, stability of the repeated sequences of *msp1α* during acute and persistent infection in the calf was demonstrated.

Similar results were obtained by Palmer et al. (2001) when studying anaplasmosis in chronically infected herds. These authors detected stable strains between 525 and 719 days after experimental infection and in animals naturally infected by *A. marginale*.

The genotypic change was then highlighted as an infrequent event, and it was hypothesized that differentiated forms of transmission would play an essential role in diversity within the herd (Palmer et al., 2001). Even after the acute phase, the analysis of the samples obtained 90 days after inoculation allowed the observation of the stability of the genotypes. This reinforces other important elements, such as the introduction and contact with other animals, a factor emphasized by, although these authors also observed the stability of lineages in individually analyzed grazing animals.

In this study, the positivity of the intestines, salivary glands and saliva analyzed suggests that the AmRio1 strain can be transmitted biologically by *R. microplus* ticks. The results showed positivity in the saliva samples, even after the need to dilute them to reach a volume of 200 µl. This shows that even after dilution there were a large number of targets. Probably the large number of targets is due to the significant replication of *A. marginale* in tick cells.

It is possible genetic diversity of *A. marginale* originates during replication in the midgut and salivary glands of vector ticks because this agent performs binary fission forming large vacuoles, which may contain thousands of organisms (Kocan et al., 1993; Kocan, 1986; Kocan et al., 2004). This large-scale replication could induce "error" and a possible predominance of a mutant strain after selective pressure. The occurrence of changes in repeated sequences is based on a process of coevolution between the pathogen and vector (de la de la Fuente et al., 2001b).

In this study, there was no diversity of *msp1α* when the organs and saliva of vector ticks were analyzed. Similarly, Palmer et al. (2001) obtained only a single genotype with replicates of identical structure of *msp1α* to the strains initially inoculated in bovines. According to (Kocan et al., 1993), the initial replication occurs inside the intestinal epithelium, whereas replication and final development of infectivity take place in the salivary gland.

The ticks used in this study were fed the blood of the experimentally infected calf, but only one generation of ticks was analyzed, and the calf was not exposed to other strains of ticks during the acute phase. Hence, the only possibility of evolution during the stable period was replication in cells of one generation of the vector and subsequent exposure to the vertebrate host immune system because the evolution of MSP1a is probably influenced by the selective pressure exerted by the immune system of the host, interactions between pathogen and cell receptors and other factors required for parasite survival (de la de la Fuente et al., 2001b).

There is an epitope sensitive to neutralization in the repeated sequences of MSP1a (Oberle et al., 1988; Palmer et al., 1987), suggesting that the protein may experience selective pressure exerted by the host immune system, leading to the emergence of new strains of *A. marginale*.

Although the animal in this study was immunocompetent, no change was observed in the repeated sequences during acute and persistent infection. The same result was observed in ticks. In addition, the selective pressure in ticks tends to be like the pressure that *A. marginale* suffers in vitro, that is, lower than in vertebrate hosts.

5. Conclusions

Changes in parameters and clinical signs evaluated indicate that the *A. marginale* strain, AmRio1, is pathogenic. Furthermore, this strain exhibited no change in its repeated sequences of MSP1a during acute infection and chronic infection. *A. marginale* also did not express diversity in the sequences obtained from the DNA of organ pools and saliva of ticks positive for *msp1α*. These results reinforce the idea of the low frequency of occurrence of genotypic alteration. The positivity of samples from ticks suggests the biological transmission of AmRio1 by *R. microplus*.

Conflict of interest

The authors declare that there is no conflict of interest with respect to the research, authorship, and/or publication of this article.

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