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Short communication

Low genetic diversity associated with low prevalence of *Anaplasma marginale* in water buffaloes in Marajó Island, Brazil

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ABSTRACT

The rickettsia *Anaplasma marginale* is the etiologic agent of bovine anaplasmosis, an important tick-borne disease affecting cattle in tropical and subtropical regions of the world. In endemic regions, the genetic diversity of this pathogen is usually related to the high prevalence of the disease in cattle. The major surface protein 1 alpha (MSP1a) has been used as a marker to characterize the genetic diversity and for geographical identification of *A. marginale* strains. The present study reports the characterization of *A. marginale* MSP1a diversity in water buffaloes. Blood samples were collected from 200 water buffaloes on Marajó Island, Brazil where the largest buffalo herd is located in the Western hemisphere. Fifteen buffaloes (7.5%) were positive for *A. marginale msp1α* by PCR. Four different strains of *A. marginale* with MSP1a tandem repeat structures (4-63-27), (162-63-27), (78-24-24-25-31) and (τ-10-10-15) were found, being (4-63-27) the most common. MSP1a tandem repeats composition in buffalos and phylogenetic analysis using *msp1α* gene showed that the *A. marginale* strains identified in buffaloes are closely related to *A. marginale* strains from cattle. The results demonstrated low genetic diversity of *A. marginale* associated with low bacterial prevalence in buffaloes and suggested that buffaloes may be reservoirs of this pathogen for cattle living in the same area. The results also suggested that mechanical transmission and not biological transmission by ticks might be playing the major role for pathogen circulation among water buffaloes in Marajó Island, Brazil.

Introduction

Anaplasma marginale (Rickettsiales: Anaplasmataceae) is the most prevalent pathogen transmitted by ticks worldwide, distributed on the six continents and responsible for high morbidity and mortality in cattle in temperate, subtropical, and tropical regions (Vidotto et al., 1998; Kocan et al., 2010). Bacteria of the genus *Anaplasma* are obligate intracellular pathogens that can be transmitted biologically by ticks, mechanically by hematophagous insects and blood-contaminated fomites and less frequently transplacentally (Kocan et al., 2010).

The global distribution and high pathogenicity of *A. marginale* is due to the diversity and genetic variability of this bacterium (de la Fuente et al., 2007). This pathogen has over 20 proteins capable of inducing protective immunity (Suarez and Noh, 2011) from which major surface proteins (MSPs) have been extensively characterized (Kocan et al., 2010). Among the major surface proteins (MSPs), special attention has been directed to MSP1a because it is involved in the interaction of the bacterium with vertebrate and invertebrate host cells (de la Fuente et al., 2010). Several strains of *A. marginale* have been identified worldwide and these strains differ in their morphology, MSP1a amino acid sequence, antigenic characteristics, and ability to be transmitted by ticks (de la Fuente et al., 2007; Estrada-Peña et al., 2009; Cabezas-Cruz et al., 2013).

The primary host for *A. marginale* is cattle, but other ruminants such as deer and buffaloes can also be infected (Kocan et al., 2010). Approximately 300,000 buffaloes are geographically isolated on Marajó Island, Brazil, representing the largest buffalo herd in the Western hemisphere, and these animals have been used as a primary source of meat, milk, and leather, besides being used to plow the land and to transport people and crops (IBGE, 2012). Serological

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and molecular detection of *A. marginale* in water buffaloes in Brazil have shown a prevalence of 49.0% and 5.4%, respectively (Silva et al., 2014). However, although the *A. marginale msp1α* genetic diversity has been characterized in Brazilian cattle (de la Fuente et al., 2007; Estrada-Peña et al., 2009; Cabezas-Cruz et al., 2013; Pohl et al., 2013), a similar study has not been conducted in buffaloes.

In this study, we characterized the *A. marginale msp1α* genetic diversity in naturally infected water buffaloes on Marajó Island, Brazil. The results demonstrated low genetic diversity of *A. marginale* associated to low prevalence of the bacteria in water buffaloes and suggested that buffaloes may be a reservoir of this pathogen for cattle living in the same area. The results also suggested that mechanical transmission and not biological transmission by ticks might be playing an essential role for pathogen circulation among water buffaloes in Marajó Island, Brazil.

Materials and methods

Experimental design and study site

A cross-sectional molecular study was conducted sampling buffalo herds in four provinces of Marajó Island, Brazil (Soure, Salvaterra, Muaná, and Chaves) between January and December 2012. The Marajó Island hosts the largest water buffalo population in the Western hemisphere. The vegetation on this island is predominantly provided by the Amazon tropical rainforest (Furtado et al., 2009). The buffaloes are vaccinated against brucellosis and foot-and-mouth disease, but endo and ectoparasite control is rarely used. Large areas of bog and grassland along the floodplains of rivers are found on Marajó Island (Furtado et al., 2009). These animals are reared using an extensive system. The main tick species found on animals are *Amblyomma cajennense*, *Rhipicephalus (Boophilus) microplus*, *Dermacentor nitens* and *A. maculatum*. These tick species can be found on buffaloes with low infestation rates throughout the year (Silva et al., 2014).

Sample collection and DNA extraction

Two hundred female water buffaloes with approximately 3 years of age were randomly selected in at least three farms from each province included in the study. Blood samples were collected from the caudal or jugular veins of individual animals. DNA extraction was performed using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA) following manufacturers recommendations.

A. marginale msp1α PCR and DNA sequencing

The primers 1733F (5′ TGTGCTTATGGCAGACATTTCC 3′), 3134R (5′ TCACGGTCAAAACCTTTGCTTACC 3′), and 2957R (5′ AAACCTTGTAGCCCCAACTTATCC 3′) were used to amplify *A. marginale msp1α* as reported previously (Lew et al., 2002). Briefly, primers 1733F and 3134R were used in the first PCR amplification, while 1733F and 2957R were used in a nested-PCR reaction. For both reactions, 12.5 μl PCR Master Mix (Qiagen, Valencia, CA, USA), 20 pmol of each primer and 5 μl genomic DNA (first reaction) were used in a final volume of 25 μl. For the second reaction 1 μl of the DNA amplified in the first reaction was used as template. Control reactions were performed in a similar way but without DNA added to it. After the PCR reaction, amplicons were purified with the Silica Bead DNA Gel Extraction Kit (Fermentas Life Sciences, Sao Paulo, Brazil) following manufacturer's instructions and sequenced. The *A. marginale msp1α* sequences obtained in this study from water buffaloes are available in GenBank with accession numbers KJ575588–KJ575602.

A. marginale msp1α sequence analysis

A microsatellite is located at the *msp1α* 5′ untranslated region (UTR) between the putative Shine-Dalgarno (SD) sequence (GTAGG) and the start codon (ATG). The general microsatellite structure is as previously reported GTAGG (**G/A TTT**)*m* (**GT**)*n* **T** ATG (Estrada-Peña et al., 2009) where microsatellite sequence is in bold letters. The SD-ATG distance was calculated according to the equation $(4 \times m) + (2 \times n) + 1$. Based on the structure of this microsatellite eleven genotypes (named with Latin alphabet letters from A to K) of *A. marginale msp1α* have been previously identified (Estrada-Peña et al., 2009; Cabezas-Cruz et al., 2013). Theoretical translation of *msp1α* DNA into amino acid sequences was performed using the Expasy Translation Tool (http://expasy.hcuge.ch/tools/dna.html). Tandem repeats were identified and named according to the nomenclature proposed by de la Fuente et al. (2007) and updated by Cabezas-Cruz et al. (2013). Tandem repeat sequences were aligned using MUSCLE (v3.7) (Edgar, 2004). Codon based alignment was performed using the codon suite server (Schneider et al., 2007). Detection of selection pressure on individual codons was calculated using two methods, single likelihood ancestor counting (SLAC) and fixed effects likelihood (FEL) implemented in Datamonkey webserver (Delport et al., 2010). Positive or negative selection was assigned to codons where $\omega = dN$ (non-synonymous substitutions)/dS (synonymous substitutions) ratio was higher or lower than 1, respectively. As recommended in Datamonkey webserver (Delport et al., 2010), only sites with p-value < 0.25 were considered to be under selection.

Phylogenetic analysis

For *msp1α* phylogenetic analysis, nucleotide sequences were aligned with MUSCLE (v3.7) configured for high precision (Edgar, 2004) followed by removal of the ambiguous regions with Gblocks (v0.91b) (Castresana, 2000). The phylogenetic tree was constructed using the neighbor joining method implemented in Neighbor from the PHYLIP package (v3.66) (Felsenstein, 1989). Internal branch confidence was assessed by the bootstrapping method using 1000 bootstrap replicates. Sequences of *A. marginale msp1α* previously reported in cattle from Brazil and the USA were obtained from Genebank and used as outgroups.

Results and discussion

Low prevalence of *A. marginale* was recently reported in buffaloes in Marajó Island, Brazil, using the major surface antigen 5 (*msp5*) gene marker (Silva et al., 2014). The results obtained in the present study using *msp1α* agreed with those reported by Silva et al. (2014) and showed 7.5% (15 positive samples) prevalence of *A. marginale* in water buffaloes from Marajó Island, Brazil. This prevalence could be considered low when compared with the prevalence of *A. marginale* in cattle from Brazil. For example, using *msp1α*, a recent study showed 70% prevalence of *A. marginale* in a herd of Brazilian cattle (Pohl et al., 2013). Water buffaloes with clinical anaplasmosis were not registered in the present study. The pathogenic significance of *A. marginale* for water buffaloes remains to be elucidated, but the fact that buffaloes can carry *A. marginale* raise concerns regarding the role of this species as reservoirs of *A. marginale* for cattle living in the same area (Silva et al., 2014). Phylogenetic analysis using *msp1α* show that *A. marginale* strains found in buffaloes are closely related to strains isolated previously from cattle in Brazil (Fig. 1A), suggesting that buffaloes can be infected with the same strains that infect cattle and thus buffaloes could constitute reservoir hosts for *A. marginale* in cattle. Further research is needed to elucidate the role of water buffaloes as reservoir hosts for *A. marginale* in cattle in this or other regions where both species share the same space.

The *gene msp1α* has been extensively used for the characterization of the genetic diversity of *A. marginale* in cattle (Palmer et al., 2001; de la Fuente et al., 2007; Ruybal et al., 2009; Estrada-Peña

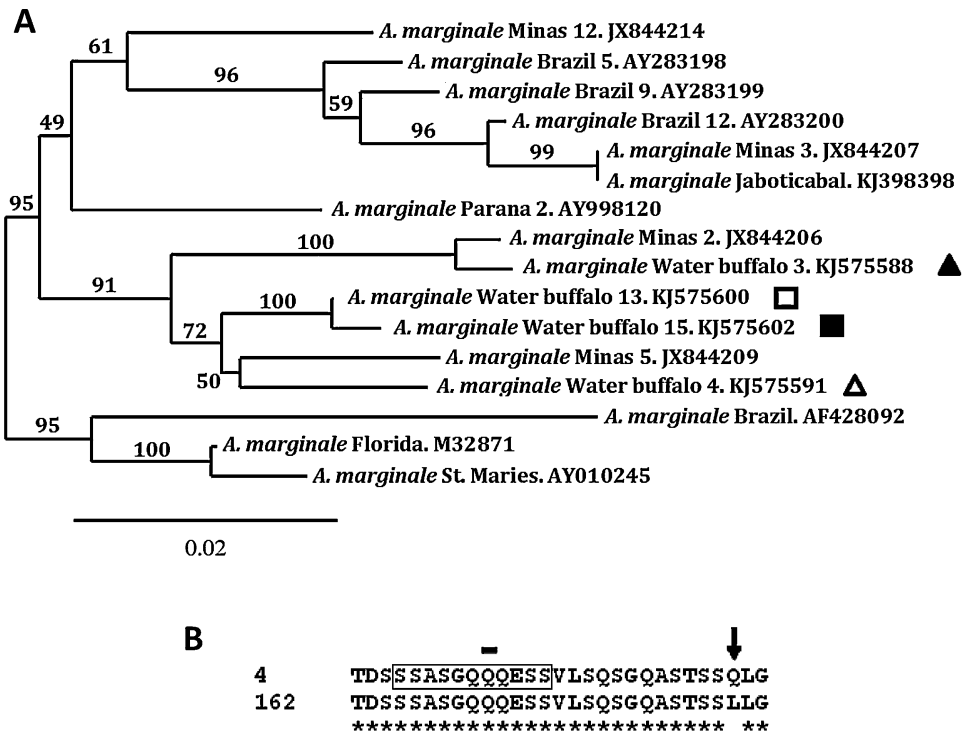

Fig. 1. Characterization of *A. marginale msp1α* sequences. (A) Neighbor joining phylogenetic tree of *A. marginale msp1α*. The tree was constructed using the neighbor joining method with *A. marginale msp1a* sequences from strains identified in water buffaloes and cattle. Bootstrap values are represented as percent on internal branches (1000 replicates). The GenBank accession numbers of the respective sequences used for the phylogenetic analysis are shown. The four different *A. marginale* strains obtained from water buffaloes in this study are shown (together with tandem repeat structure in parenthesis) as Water buffalo 3 (78, 24, 24, 25, 31) (black triangle); Water buffalo 13 (4, 63, 27) (white square); Water buffalo 15 (162, 63, 27) (black square) and Water buffalo 4 (τ, 10, 10, 15) (white triangle). (B) Amino acid differences between tandem repeats 4 and 162 and position evolving under negative selection. The one letter code is used for the different amino acids of the tandem repeats. Conserved amino acid positions are highlighted with asterisks. Substitution of glutamine (Q) in tandem repeat 4 by leucine (L) in tandem repeat 162 is show with an arrow. Amino acid at position 10 (-) evolving under negative selection ($p < 0.25$ using both FEL and SLAC methods) and residues of the immunodominant B-cell epitope (Garcia-Garcia et al., 2004) (box) are also shown.

et al., 2009; Cabezas-Cruz et al., 2013; Pohl et al., 2013) but little is known about the genetic diversity of *A. marginale* in other species of ungulates, including buffaloes (de la Fuente et al., 2004). In order to determine the genetic diversity of *A. marginale* infecting buffaloes, we sequenced the 15 *msp1α* positive samples that were obtained in the present study (Table 1). The results showed that the genetic diversity of *A. marginale msp1α* in buffaloes from Marajó Island is low, with only four different strains identified, showing the microsatellite genotype E (Table 1). In contrast, the results by Pohl et al. (2013) in cattle showed, in 13 sequenced samples, 8 different strains of *A. marginale* with four different microsatellite genotypes (B, D, E and G). Three possibilities could be considered in order to explain the low genetic diversity of *A. marginale* in buffaloes in Marajó Island: (a) in bovine anaplasmosis endemic regions, low genetic diversity of *A. marginale msp1α* has been related to tick absence (Ruybal et al., 2009). Most of the sampled buffaloes in this study were raised on submerged wetlands, where tick attachment is rare (Silva et al., 2014) and thus tick infestation rates are low and transmission of *A. marginale* is an unlikely event; (b) cattle movement has been proposed as a source of genetic diversity in *A. marginale* worldwide (de la Fuente et al., 2007). In Marajó Island, the entry of new buffaloes is prohibited, limiting the possibility of the introduction of new strains of *A. marginale* and consequently bacterial genetic diversity in this area. This phenomenon is in agreement with the fact that cattle movement is limited in Australia where only one strain of *A. marginale* has so far been identified in cattle (Lew et al., 2002); (c) finally, it could be argued that *A. marginale* was just recently introduced in this buffalo herd, which will result in low genetic diversity. Low genetic diversity of *msp1α* was reported in a previously uninfected cattle herd where only a single *msp1α* genotype was found (Palmer et al., 2001).

Table 1
Organization of MSP1a tandem repeats in *A. marginale* strains identified in water buffaloes.

A. marginale strain identification	No. of animals infected with this strain
Brazil/Marajó Island/E – (4, 63, 27)	9
Brazil/Marajó Island/E – (78, 24^2, 25, 31)	3
Brazil/Marajó Island/E – (τ, 10^2, 15)	2
Brazil/Marajó Island/E – (162, 63, 27)	1

Strain identification is based on *msp1α* and includes Country/Locality/microsatellite genotype – (tandem repeats structure). Superscripts represent the number of times that a tandem repeats are repeated. The new MSP1a tandem repeat 162 was named following the system proposed by de la Fuente et al. (2007) and updated by Cabezas-Cruz et al. (2013).

Despite the low genetic diversity observed for *A. marginale* in buffaloes, evidence of genetic diversification was found. The *A. marginale* strains obtained from buffaloes in this study had between 3 and 5 MSP1a repeat sequences (Table 1). Tandem repeat 162 was found for the first time in this study (Table 1 and Fig. 1B). Tandem repeats 162 and 4 only differ in one amino acid at position 27 with glutamine (Q) in tandem repeat 4 and leucine (L) in tandem repeat 162 (Fig. 1B). In addition, the amino acid Q in tandem repeat 4 is encoded by the codon CAA and a single mutation to uracile in the second adenine of the CAA codon will result in the codon CUA which encodes for the amino acid L in tandem repeat 162. This finding suggested that the tandem repeat 162 may have originated recently from tandem repeat 4, providing evidence for genetic diversification of *A. marginale* in water

buffaloes. In agreement with this hypothesis, the phylogenetic analysis using *msp1α* indicated that the strain Water buffalo 15 (162, 63, 27) possibly evolved from strain Water buffalo 13 (4, 63, 27) (Fig. 1A). In order to determine which selective pressures could be triggering MSP1a diversification in *A. marginale* from buffaloes, the ratio ω was calculated showing that codon at position 10 from tandem repeat 4 was evolving under negative selection (Fig. 1B). Interestingly, this amino acid position is present in an immunodominant B-cell epitope described before for *A. marginale* MSP1a (Garcia-Garcia et al., 2004) (Fig. 1B). These results suggested that this tandem repeat which was present in the most common strain of *A. marginale* found in buffaloes (Table 1) may be under selective pressure by the host immune system (Garcia-Garcia et al., 2004).

Some of the tick species found infesting buffaloes such as *Rhipicephalus* and *Dermacentor* spp. have been recognized as vectors of *A. marginale* (Kocan et al., 2010). However, the low tick infestation rates found in buffaloes in the study area suggested that mechanical and/or transplacental transmission could be playing an important role in *A. marginale* transmission in this buffalo herd. The sucking lice, *Haematopinus tuberculatus* was implicated recently in *A. marginale* transmission and outbreaks of this lice species have been reported in buffaloes (Da Silva et al., 2013). Differential tick transmission fitness has been found among different *A. marginale msp1α* genotypes (Palmer et al., 2004). Considering that ticks may not be playing an important role in transmission among buffaloes in the study site, the most common strain found in water buffaloes may be adapted to mechanical or transplacental transmission. In agreement with these findings, 60% of the *A. marginale* MSP1a tandem repeats obtained here presented the amino acid glycine (G) at position 20 and this amino acid was in at least one of the MSP1a repeats in all the *A. marginale* strains. The negatively charged amino acids aspartic acid (D) and glutamic acid (E) at position 20 were shown to be essential for the binding of MSP1a to tick cells while with a G at this position no binding was observed (de la Fuente et al., 2003). These amino acids affect MSP1a conformation and these conformational changes were suggested to affect *A. marginale* transmission by ticks (Cabezas-Cruz et al., 2013).

Conclusions

In this study, the genetic diversity of MSP1a in *A. marginale* was characterized in water buffaloes. The *A. marginale* genetic diversity was low in buffaloes and correlated with the low bacterial prevalence in this species. One major factor that could be contributing to this low genetic diversity is the ecology of the studied area, which is not suitable for ticks thus reducing the probability for pathogen biological transmission. Mechanical transmission by hematophagous Diptera could be playing a major role in the transmission of *A. marginale* in the study site. Evidence was found to support the hypothesis that MSP1a is under selective pressure by the host immune system in buffaloes. Finally, water buffaloes may serve as reservoir hosts of *A. marginale* for cattle. These results expanded our knowledge of *A. marginale* strains and provided additional support for the role of MSP1a in pathogen evolution and transmission.

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