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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