Short communication

Infection of water buffalo in Rio de Janeiro Brazil with Anaplasma marginale strains also reported in cattle

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A B S T R A C T

Anaplasma marginale is the most prevalent pathogen of cattle in tropical and subtropical regions of the world and causes the disease bovine anaplasmosis. The importance of water buffalo in the world economy is increasing. In addition, while water buffalo may serve as a reservoir host for A. marginale, the susceptibility of this host for A. marginale cattle strains in Brazil has not been reported. The major surface protein 1 alpha (msp1α) gene has been shown to be a stable genetic marker for identification of A. marginale strains. Herein, we analyzed blood samples from 200 water buffalo and identified the A. marginale strains in an endemic area of Rio de Janeiro, Brazil, where ticks were present and water buffalo and cattle co-mingled. Ticks that were feeding on the study buffalo were collected and identified. The prevalence of A. marginale in water buffalo in this study was low (18%). Sequence analysis of the msp1α gene demonstrated the presence of 8 different A. marginale strains. Two A. marginale strains in the water buffalo, (α-β-β-β-γ) and (α-β-β-γ), were similar to those reported in cattle from nearby regions. The results of this study suggested that water buffalo in this region are naturally infected with the same strains of A. marginale found in cattle.

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1. Introduction

Anaplasma marginale is a tick-borne pathogen of the family Anaplasmataceae, order Rickettsiales. This gram negative bacterium is an obligate intraerythrocytic pathogen causing bovine anaplasmosis, a disease affecting cattle in temperate, subtropical and tropical regions of the world (revised in Aubry and Geale, 2011). Not only cattle, but also other domestic and wild ruminants such as water buffalo (Bubalus bubalis) can become persistently infected and carriers of A. marginale (Rajput et al., 2005). However, our knowledge regarding A. marginale transmission to wild ruminants is based mostly on laboratory experimentation (mostly blood transfusion studies), but there are only few field studies to validate this experimental transmission in natural environments (revised in Aubry and Geale, 2011). Cattle and water buffalo are usually raised together in Brazil.

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(Silva et al., 2014a). Therefore, from an epidemiological point of view one relevant question that remains unanswered is whether the same strains of *A. marginale* can infect cattle and water buffalo.

Analyses of *msp1α* (major surface protein 1 alpha) gene sequences have allowed the identification of *A. marginale* strains worldwide (Cabezas-Cruz et al., 2013) and despite the *msp1α* genetic diversity, this gene is used as a stable genetic marker conserved during acute and persistent rickettsiemia in cattle and also during multiplication in ticks (revised in Aubry and Geale, 2011).

Brazil is considered an endemic region for *A. marginale* in cattle (Vidotto et al., 2006; Pohl et al., 2013) and recently, infection of water buffalo with *A. marginale* was reported in Brazil and their role as potential natural reservoirs for *A. marginale* was suggested (Silva et al., 2014a). Here we report an epidemiological context where the same strains of *A. marginale* previously reported in cattle were found infecting water buffalo.

2. Materials and methods

2.1. Study site and water buffalo population

The present study was carried out in Rio de Janeiro, Brazil, throughout the year 2011. The vegetation is predominantly Atlantic Forest (tropical forest). The water buffalo in this region are vaccinated against brucellosis and foot and mouth disease. Epidemiological relevant characteristics of the water buffalo population in Rio de Janeiro: (i) it is a relatively small population (Corrêa et al., 2012), (ii) it is in close contact with cattle (Corrêa et al., 2012), (iii) it is infested with tick vectors of *A. marginale* in cattle and endo- and ectoparasite control is rarely used (Corrêa et al., 2012) and (iv) it is located nearby geographic regions such as Paraíba (Vidotto et al., 2006), Northeast Argentina (Ruybal et al., 2009) and Minas Gerais (Pohl et al., 2013) where *A. marginale* has been previously characterized in cattle using *msp1α*, allowing for sequence comparison.

2.2. Samples collection, DNA extraction and *msp1α* PCR

Blood samples were collected from each of 200 water buffalo. Whole blood samples were collected from the tail or jugular veins into EDTA blood collection tubes. Additionally, 40 ticks that were feeding on the water buffalo included in the study were collected and identified. The ticks were classified and stored in 70% alcohol for further DNA extraction. Total DNA from blood samples or ticks was extracted using the DNeasy® Blood & Tissue kit (Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. The DNA concentration from each sample was quantified using a NanoDrop spectrophotometer and stored at −20 °C. A semi-nested PCR (nPCR) was used to amplify the *msp1α* sequence as previously reported (Lew et al., 2002). PCR products were directly sequenced using an ABI 3130 sequencer (Applied Biosystems, USA) and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Both the sense and antisense strands of each PCR-amplified product were sequenced, and a consensus sequence was obtained for each amplified PCR product.

2.3. Quantitative PCR for detection and quantitation of *A. marginale*

The real-time PCR reaction was performed according to Carelli et al. (2007) with modifications to amplify a *msp1α* segment. The primers forward: 5′-TTGCCAAGCAGCACGTT-3′ and reverse: 5′-CTCCGAGCACTTGCAT-3′ and the probe (6FAM-5′-TTCGTCCTAATACCTCCAGGCT TTCCAT-3′-BHQ1) were used in a reaction containing 100 ng of genomic DNA using TaqMan® Gen Expression Master Mix (Qiagen, Madison, USA). The amplification reactions were performed in a CFX96 Thermal Cycler (BioRad, Hercules, CA, USA). All samples were tested in triplicate. Quantification of the copy number of target DNA/μL was performed using the IDT pSMARTplasmids (Integrated DNA Technologies, Coralville, Iowa, USA), which contained the *A. marginale* *msp1α* target gene. Serial dilutions were made to obtain standards with different concentrations of plasmid DNA containing the target sequence (2.0 × 10⁷ copies/μL to 2.0 × 10⁰ copies/μL). The plasmid copy number was determined according to the following formula: [(Xg/μL DNA)/(plasmid size (bp) × 660)] × 6,022 × 10²⁴ plasmid copies/μL. Ultra-pure sterile water (Qiagen, Madison, Wisconsin, USA) and DNA obtained from blood samples of cattle known to be negative for *A. marginale* were used as negative controls. Once the bacterial copy number was determined for 100 ng of template DNA, the number of organisms per ml of whole blood was calculated.

2.4. *A. marginale* *msp1α* sequence analysis

A microsatellite located at the 5′-untranslated region (UTR) of the *msp1α* gene between the putative Shine-Dalgarno (GTAGG) sequence and the translation initiation codon (ATG) was previously identified in the *msp1α* sequences. The microsatellite structure is GTAGG (G/ATT)m (GT)n T ATG. The SD-ATG distance was calculated according to the formula: (4 × m) + (2 × n) + 1. The *msp1α* genotypes based on the above microsatellite were revised in Cabezas-Cruz et al. (2013). The tandem repeat of the *msp1α* amino acid sequences were classified following the numbering by Cabezas-Cruz et al. (2013).

2.5. Phylogenetic analysis

The phylogenetic analyses were conducted with *msp1α* amino acid sequences aligned with MAFFFT (v7) configured for the highest accuracy (Katoh and Standley, 2013). After alignment, regions with gaps were removed from the alignment. Phylogenetic trees were reconstructed using maximum likelihood (ML) and neighbor joining (NJ) as implemented in PhyML (v3.0 aLRT) (Anisimova and Gascuel, 2006) and PHYLIP (v3.66) (Felsenstein, 1989), respectively. The reliability for the internal branches of ML was assessed using the bootstrapping method (1000 bootstrap replicates) and the approximate likelihood ratio test (aLRT-SH-Like) (Anisimova and Gascuel, 2006). Reliability
Table 1
Organization of A. marginale MSP1α tandem repeats and bacteremia in strains identified in buffaloes.

<table>
<thead>
<tr>
<th>Strain identification and structure of MSP1α tandem repeats</th>
<th>Isolated from (number of the sample)</th>
<th>Bacteria level</th>
</tr>
</thead>
<tbody>
<tr>
<td>E–(τ, 10^3, 15)</td>
<td>Water buffalo (16)</td>
<td>2.71 × 10^7</td>
</tr>
<tr>
<td>E–(4, 10, 3)</td>
<td>Water buffalo (17)</td>
<td>7.86 × 10^4</td>
</tr>
<tr>
<td>E–(τ, 10^3, 15)</td>
<td>Water buffalo (18)</td>
<td>3.45 × 10^6</td>
</tr>
<tr>
<td>E–(α, β, 1, Γ)</td>
<td>Water buffalo (19)</td>
<td>5.12 × 10^6</td>
</tr>
<tr>
<td>E–(α, β, 2, Γ)</td>
<td>Water buffalo (20)</td>
<td>3.23 × 10^10</td>
</tr>
<tr>
<td>E–(τ, 10^3, 15)</td>
<td>Water buffalo (21)</td>
<td>2.68 × 10^6</td>
</tr>
<tr>
<td>E–(4, 63^2)</td>
<td>Water buffalo (22)</td>
<td>5.12 × 10^4</td>
</tr>
</tbody>
</table>

Strain identification is based on MSP1α and includes microsatellite genotype and tandem repeats structure. Superscripts represent the number of times that a tandem repeat is repeated. New tandem repeats are underlined.

for the NJ tree was assessed using bootstrapping method (1000 bootstrap replicates). Graphical representation and editing of the phylogenetic trees were performed with TreeDyn (v 198.3).

3. Results

3.1. A. marginale prevalence and msp1α sequence analyses

Twenty buffalo blood samples (10%) resulted positive for A. marginale DNA by msp1α quantitative PCR. The bacteremia of A. marginale, measured as the number of msp1α copies/ml in the blood of positive buffalo, ranged from 5.12 × 10^4 to 3.23 × 10^10.

The msp1α gene was amplified and sequenced in 7 and 4 samples from water buffalo and ticks, respectively. Eight different A. marginale strains were identified after MSP1α amino acid sequence analysis (Table 1). The strain (τ-10-10-15) was the most common and was detected in 3 water buffalo. Water buffalo carrying A. marginale strains with msp1α structure (τ-10-10-15), (α-β-β-β-Γ) and (α-β-β-β-Γ) presented the highest bacteremia (Table 1). The strain (α-β-β-β-Γ) was found in a water buffalo and also in a R. microplus tick collected from the same animal. Three new msp1α tandem repeat sequences were found in A. marginale strains present in R. microplus (Nos. 163: VDSSAGDQQQQSESSQGGDSSTSSQLG and 164: TDSSAGQDDQQQQSSQGGDSSTSSQLG) and A. cajennense (No. 165: TDSSASGGQQQSESSLPSGQASTSSQGS). We could not establish whether the strains found in ticks were in the blood meal or replicating in the gut or salivary glands of the ticks.

The A. marginale strains identified in water buffalo and ticks from Rio de Janeiro (Table 1) were compared with those reported in Minas Gerais (Pohl et al., 2013), Parana (Vidotto et al., 2006) and Northeastern Argentina (Ruybal et al., 2009) (Fig. 1). The strains (α-β-β-β-Γ) and (α-β-β-Γ), were previously reported in cattle from Northeastern Argentina and Minas Gerais, respectively. The strain (α-β-β-β-β-Γ), which is phylogenetically related to (α-β-β-β-Γ) and (α-β-β-Γ), was not identified in buffalo but was previously found in cattle from Parana and Northern Argentina (Figs. 1 and 2). Two other related strains, (τ-10-10-15) and (τ-10-15), were isolated from buffalo and cattle, respectively (Figs. 1 and 2). A. marginale strains from cattle and water buffalo formed two phylogenetic clusters related to the presence of tandem repeats α and τ, but independently from the host species from where they were isolated (Fig. 2).

No all the water buffalo were infested by ticks, but only 40% (20%). 40 ticks were randomly collected from these water buffalo (One tick per animal) and classified. The ticks belonged to 3 different species: R. microplus, D. nitens and A. cajennense, being R. microplus and A. cajennense the more represented.

4. Discussion

A. marginale is a pathogen that infects a wide variety of ruminant hosts with some of these species acting as reservoir hosts. A reservoir can be defined as “one or more epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population” (revised in Aubry and Geale, 2011). Our results demonstrated that the same strains of A. marginale can be found in water buffalo and cattle. This findings suggests that water buffalo, coexisting with cattle, may play a role as a reservoir host which should be investigated in the future. Natural infection of water buffalo with A. marginale has been detected by light microscopy (Rajput et al., 2005), ELISA, IFAT (Silva et al., 2014b) and PCR (Silva et al., 2014a). In this study, detection of natural infection of water buffalo with A. marginale was carried out with quantitative

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PCR using the *msp1α* gene. In agreement with previous studies using molecular diagnostic (Silva et al., 2014a), we found a low prevalence of *A. marginale* (10%) in water buffalo. This prevalence is lower than the prevalence reported in cattle in endemic areas like Minas Gerais, Brazil (Pohl et al., 2013). However, using serological diagnostic, the *A. marginale* prevalence found in water buffalo from Brazil is higher, such as 50% or 63% depending of the technic used, IFAT or ELISA, respectively (Silva et al., 2014b). This difference in the percent of prevalence using molecular or serological tests may be due to low levels of organisms in the blood of chronically infected animals difficult to detect by PCR, to animals that resolved the infection but still have detectable antibody levels or to non-specificity of the above serologic tests.

By using *msp1α* as a stable genetic marker we demonstrated that the *A. marginale* strains isolated from water buffalo and cattle in nearby areas are closely related or are the same. In the present study, the *A. marginale* strain (α-β-β-β-Γ) was identified in water buffalo. This strain (α-β-β-β-Γ) seems to be highly transmissible, it has been previously isolated from cattle in Argentina (Ruybal et al., 2009), Mexico (Almazán et al., 2008) and Taiwan (GeneBank accession number FJ188387). In fact, this strain constitutes one of the most common *A. marginale* strains worldwide (Cabezas-Cruz et al., 2013). In addition, this strain was found to be highly pathogenic and it was isolated during anaplasmosis outbreaks in Argentina (Ruybal et al., 2009) and Mexico (Almazán et al., 2008). A related *A. marginale* strain (α-β-β-Γ), was identified in the present study in water buffalo and also in 1 day old calf from Minas Gerais, suggesting transplacental transmission of this strain in cattle (Pohl et al., 2013). The same strain was found in Mexico (GeneBank accession number JN564639) (Cabezas-Cruz et al., 2013). The *A. marginale* strain (τ-10-10-15) was the most common strain in the studied buffalo population, infecting animals with variable levels of bacteremia. A similar *A. marginale* strain with genotype (τ-10-10-15) was reported in cattle from Parana, Brazil (Vidotto et al., 2006). The predominant genotype, based on the *msp1α* microsatellite, was E and also one strain was found that presented genotype G. Genotype E was predominant in cattle infected with *A. marginale* in Minas Gerais, Brazil (Pohl et al., 2013).

In summary, these results show that water buffalo can be naturally infected with the same strains of *A. marginale*.
that infect cattle in nearby regions, suggesting that infection chains may be established between these two species. Further studies are needed to study the potential of water buffalo as natural reservoirs of *A. marginale*.

**Conflict of interest statement**

None of the authors of this work have a financial or personal relationship with other people or organizations that would inappropriately influence or bias the content of this paper.

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**References**


