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Short Communication

Molecular characterization of *Anaplasma marginale* in ticks naturally feeding on buffaloes

Jenevaldo Barbosa da Silva [a,*], Adivaldo Henrique da Fonseca [b], José Diomedes Barbosa [c]

[a] Departamento de Patologia Veterinária, Universidade Estadual Paulista, Jaboticabal, São Paulo, Brazil
[b] Departamento de Parasitologia, Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brazil
[c] Departamento de Clínica Veterinária, Universidade Federal do Pará, Castanhal, Pará, Brazil

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A B S T R A C T

Anaplasma marginale is the most prevalent pathogen transmitted by ticks in cattle in tropical and subtropical regions of the world. However, the tick species involved in the transmission of *A. marginale* in buffaloes in Brazil have not been identified. The objective of the present study was to determine the presence of *A. marginale* in ticks parasitizing water buffaloes. A total of 200 samples of *Rhipicephalus microplus*, *Dermacentor nitens*, *Amblyomma cajennense*, and *Amblyomma maculatum* were collected and tested by conventional and quantitative PCR for the presence of the *msp1a* and *msp5* genes. In the present study, 35 ticks (17.5%) were positive for *A. marginale* DNA by qPCR analysis. The positive ticks belonged to four different species: *R. microplus* (22.2%), *A. cajennense* (13.8%), *A. maculatum* (16.0%), and *D. nitens* (10.0%). Individuals of the three developmental stages (larvae, nymphs, and adults) of *R. microplus* and *A. cajennense* were found to be positive for *A. marginale*, only nymphs and adults of *A. maculatum* were found to be positive, and finally, only adults of *D. nitens* were positive for *A. marginale*. Our results suggest that *R. microplus*, *A. cajennense*, *A. maculatum*, and *D. nitens* ticks may be involved in the transmission of *A. marginale* in buffaloes. However, while *A. marginale* PCR positive ticks were recorded, this does not indicate vector competence; only that the ticks may contain a blood meal from an infected host. Additionally, the results show that the strains of *A. marginale* from buffaloes and cattle are phylogenetically related.

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1. Introduction

Anaplasma marginale is the most prevalent pathogen transmitted by ticks in the world. It is found on six continents and is responsible for high morbidity and mortality in cattle in temperate, subtropical, and tropical regions (Kocan et al., 2010). In addition to cattle, *A. marginale* rickettsia has been diagnosed in other species of domestic and wild animals, such as water buffaloes in Brazil (Silva et al., 2014).

Biological transmission by ticks is more efficient than mechanical transmission by hematophagous flies (Scoles et al., 2008). Twenty different tick species are capable of transmitting *A. marginale* and play important roles in maintaining *A. marginale* in cattle (Kocan et al., 2004). However, the role and the species of tick involved in this process have not been identified in buffaloes.

Molecular diagnostic techniques have been developed that can be used as powerful tools for the detection of *A. marginale* infections in bovine blood (Molad et al., 2006). Recently, a molecular detection study of *A. marginale* in *Hyalomma asiaticum* ticks was conducted (Zhang et al., 2013). The objective of the present study was to determine the presence of *A. marginale* in ticks that were parasitizing water buffaloes.

2. Materials and methods

2.1. Design and population studies

This work consisted of a cross-sectional molecular epidemiology study conducted from January to December 2011 in a herd of water buffaloes in the state of Pará in the northeastern region and the state of Rio de Janeiro in the southeast of Brazil. Blood and tick samples were collected from buffalo herds in four provinces of the state of Pará (Soure, Salvaterra, Muaná, and Chaves) and five provinces of the state of Rio de Janeiro (Itaguaí, Casimiro de Abreu, Cachoeiras de Macacu, Barra do Piraí, and Campos Goytacazes). The buffaloes from which the ticks were collected

* Corresponding author at: Laboratório de Imunoparasitologia, Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias FCAV-UNESP, Via de Acesso Prof. Paulo Donato Castellane s/n, 14884-900 Jaboticabal, São Paulo, Brasil.

E-mail address: Jenevaldo@hotmail.com (J.B. da Silva).

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were kept in exclusive areas with little or no contact with cattle. The buffalo inhabited predominantly tropical forests (Atlantic and Amazon forest). A total of 200 samples of ticks were analyzed. The samples were pooled, and the pool consisted of two adult ticks, five nymphs, or 10 larvae. The pools were formed by ticks of the same species that were collected from a single animal (Table 1). The ticks were identified using a taxonomic key (Barros-Battesti et al., 2006). Each adult tick corresponded to a sample, and the larvae and nymph samples corresponded to pools of five and 10 specimens, respectively.

2.2. A. marginale PCR

Tick and buffalo DNA were extracted using a 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. A hemi-nested PCR reaction was used for the detection of a 548 bp fragment in the first reaction and a 345 bp fragment in the second reaction of the *msp5* gene of *A. marginale*, according to Singh et al. (2012). A semi-nested PCR reaction was used for the *msp1α* sequence, as described by Lew et al. (2002). The reactions were performed using the primers 1733F (5′-TGTGCTTATGG CAGACATTTCC-3′), 3134R (5′-TCACGGTCAAAACCTTTGCTTACC-3′), and 2957R (5′-AAACCTTGTAGCCC CAACTTATCC-3′). The first reaction was performed in a final volume of 25 μL of a mixture containing 5 μL of genomic DNA (100 ng/μL), 12.5 μL of PCR Master Mix (Qiagen, Valencia, CA, USA), 6.5 μL of ultrapure water, and 1.6 μM of each primer. The second reaction used a final volume of 25 μL of a mixture containing 1 μL of genomic DNA amplified in the first reaction, 12.5 μL of PCR Master Mix (Qiagen, Valencia, CA, USA), 10.5 μL of ultra-pure water, and 1.6 μM of each primer. The primer pair 1733F and 3134R was used in the first reaction, and the primer pair 1733F and 2957R was used in the second reaction. PCR was performed under the following conditions: an initial denaturation at 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. The same conditions were used in the second amplification cycle, except that the annealing temperature was changed to 60 °C. The amplified products (900 bp) were subjected to horizontal electrophoresis on a 1.0% agarose gel stained with ethidium bromide (0.625 μ/ml) in TBE pH 8.0 running buffer (44.58 M Tris-base; 0.44 M boric acid; 12.49 mM EDTA). A 100 bp DNA ladder (Thermo Scientific, San Jose, CA, USA) was used for determination of the amplified product. The results were visualized and analyzed with an ultraviolet light transilluminator (2020E) coupled to image analysis software (BioRad, Hercules, CA, USA).

2.3. A. marginale msp1α quantitative PCR

Real-time PCR was performed according to Carelli et al. (2007) with modifications to amplify the *msp1α* gene. The reaction was performed with a final volume of 10 μL of a mixture containing 1 μL (100 ng/μL) of genomic DNA, 5.0 μL of TaqMan® Gene Expression Master Mix (Applied Biosystems, USA), 0.9 μL (10 μM) of each of the primers (AM-forward: 5′-TTGGCAAGGCAGCAGCT T-3′ and AM-reverse: 5′-TTCCGCGAGCATGTGCAT-3′), 0.2 μL of (10 μM) the probe (AM-probe: 6FAM-5′-TCGGTCTAACATCTCCAG GCTTTCAT-3′-BHQ1), and 2.0 μL of sterile ultrapure water (Nuclease-Free Water® Promega). The cycles were performed under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The amplification reactions were conducted in a CFX96 Thermal Cycler (BioRad, Hercules, CA, USA). All samples were tested in triplicate. Quantification of the copy number of the target-DNA/μL was performed using the psmart IDT plasmid (Integrated DNA Technologies, Coralville, Iowa, USA), which contained the target sequences for amplification of *A. marginale* DNA (*msp1α* gene). Serial dilutions were made to establish standards with different concentrations of plasmid DNA containing the target sequence (2.0×10^7 copies/μL to 2.0×10^0 copies/μL). The plasmid copy number was determined using the formula (Xg/μL DNA/[plasmid size (bp) × 660]) × 6.022 × 1023 × copies of plasmid/μL. Ultrapure sterile water (Qiagen, Madison, USA) and the DNA obtained from blood samples of cattle known to be *A. marginale* negative were used as negative controls.

Table 1
Number of samples per species and developmental stages of ticks collected from buffaloes. Molecular prevalence of *A. marginale* in qPCR by species and developmental stage ticks collected from buffaloes in Brazil, 2011.

Tick species	Developmental stage			Overall (+)
	Larva	Nymph	Adult	
R. microplus	6.6% (1/15)	20.0% (5/25)	28.0% (14/50)	22.2% (20/90)
A. cajennense	10.0% (1/10)	10.0% (2/20)	17.1% (6/35)	13.8% (9/65)
A. maculatum	0.0% (0/5)	10.0% (1/10)	33.3% (3/10)	16.0% (4/25)
D. nitens	0.0% (0/0)	0.0% (0/15)	13.3% (2/15)	10.0% (2/20)

2.4. Phylogenetic analysis

The phylogenetic analysis was performed with *msp1α* nucleotide sequences aligned with MAFFT (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 the maximum likelihood (ML), neighbor joining (NJ), and Bayesian inference (MB) methods as implemented in PhyML (v3.0 aLRT) (Guindon and Gascuel, 2003; Anisimova and Gascuel, 2006), PHYLIP (v3.66) (Felsenstein, 1989), and MrBayes (v3.1.2) (Huelsenbeck and Ronquist, 2001), respectively. The reliability for the internal branches of the ML was assessed using the bootstrapping method (1000 bootstrap replicates) and the approximate likelihood ratio test (aLRT – SH-Like) (Anisimova and Gascuel, 2006). The reliability for the NJ tree was assessed using the bootstrapping method (1000 bootstrap replicates). Additionally, 10,000 generations of Markov Chain Monte Carlo (MCMC) chains were run. For the graphical representation and editing of the phylogenetic trees, TreeDyn (v 198.3) was used (Chevenet et al., 2006).

Fig. 1. Level of tick infestation. Young Murrah buffalo exhibiting high infestation by *R. microplus* and *A. cajennense* ticks.

3. Results and discussion

In the present study, only the tick species *Rhipicephalus microplus*, *Amblyomma cajennense*, *Amblyomma maculatum*, and *Dermacentor nitens* were found to parasitize buffaloes in Brazil. We observed that most buffaloes had low tick infestation (less than 20 engorged females/animal). However, some animals, especially those aged less than 6 months, had an increased tick infestation (more than 100 engorged females/animal) (Fig. 1). Previous studies have shown that the species of ticks that parasitize buffaloes are similar to those that parasitize cattle, and approximately 20 different species, including *R. microplus*, *Amplyomma* sp., and *Dermacentor* sp., have been found to parasitize buffaloes (Miranpuri, 1988). The presence of buffaloes naturally infested with *R. microplus* and *D. nitens* has been reported in Brazil (Rocha et al., 1969). Only 4.03% of *R. microplus* ticks reach the adult stage when parasitizing buffaloes (Starke et al., 1994). Moreover, in a comparison of the level of natural infestation by *R. microplus* in cattle and buffaloes, it was observed that buffaloes had 6 times fewer adult ticks than cattle (Starke et al., 1994).

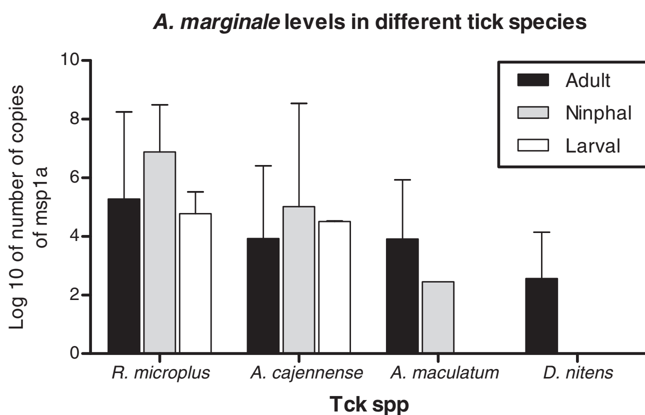

Fig. 2. Levels of *A. marginale* tick species. The number of *A. marginale* DNA copies per sample for *R. microplus*, *A. cajennense*, *A. maculatum*, and *D. nitens*.

In the present study, 35 ticks (17.5%) were positive for *A. marginale* DNA according to quantitative PCR (qPCR) assays. The positive ticks belonged to four different species: *R. microplus* (22.2%), *A. cajennense* (13.8%), *A. maculatum* (16.0%), and *D. nitens* (10.0%). *R. microplus* and *A. cajennense* ticks were positive for *A. marginale* in all three life cycle stages; in *A. maculatum*, nymphs and adults were found positive for *A. marginale*, and in *D. nitens*, only adults presented *A. marginale* positive DNA (Table 1). This is the first study showing the possible involvement of *Amblyomma* in the transmission of *A. marginale*. Twenty different species of ticks are suspected to be potential vectors of *A. marginale* worldwide (Ewing, 1981) including *R. microplus*, *Ixodes* sp., *Hyalomma* sp., and *Dermacentor* sp. The genus *Amblyomma* has not been suspected to transmit *A. marginale* (Kocan et al., 2010). In Tanzania, six tick species were detected with *A. marginale* DNA including *Amblyomma gemma*, *Rhipicephalus appendiculatus*, *Rhipicephalus compositus*, *Rhipicephalus decoloratus*, *Rhipicephalus praetextatus*, and *Rhipicephalus pulchellus* (Fyumagwa et al., 2009). Although studies have been conducted that describe the main tick species that parasitize buffaloes, no studies have evaluated their competence in the transmission of pathogens such as *Anaplasma* sp. and *Babesia* sp.

The number of *A. marginale* DNA copies per sample ranged from 2.40×10^2 to 5.89×10^{11} for the *R. microplus* tick, from 6.10×10^1 to 2.97×10^8 for *A. cajennense*, from 3.93×10^1 to 2.21×10^5 for *A. maculatum*, and from 2.79×10^2 to 4.79×10^3 for *D. nitens* (Fig. 2). The two buffaloes evaluated showed *A. marginale* DNA copy numbers of 1.23×10^2 and 3.54×10^6. For the *R. microplus* species, the qPCR results were significantly higher in the nymph stage than in larvae and adults. Although the qPCR results of *A. marginale* in ticks cannot be compared with those observed in cattle, some values were similar or greater than the values observed in clinically ill animals, especially for the *R. microplus* tick (Carelli et al., 2007). Thus, this technique may be an important tool for the detection

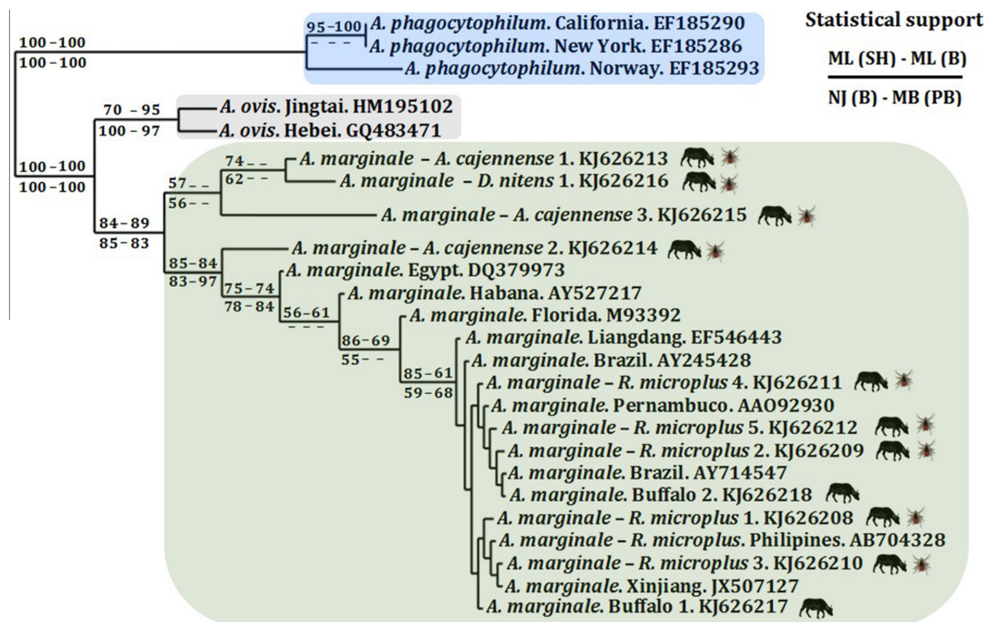

Fig. 3. Phylogenetic tree of the *msp5* gene from members of the genus *Anaplasma*. Phylogenetic analyses were conducted using the ML, NJ, and MB methods. The topologies obtained with the three methods were similar. The ML topology is provided. Nucleotide sequences from the *A. ovis*, *A. marginale*, and *A. phagocytophilum msp5* gene were used to perform the phylogenetic analysis. The numbers above and below the internal branches represent bootstrap values (ML and NJ), posterior probabilities (MB), and aLRT – SH-Like (ML) as shown (Statistical support). Only values higher than 50 are represented. The GenBank accession numbers of the sequences used in the phylogenetic analyses are also shown.

of *A. marginale* in ticks, buffaloes, cattle, and other ruminants. Because it is a highly sensitive and specific technique, qPCR for *A. marginale* can be used for both detection and comparison of the level of parasitism among animals of different species.

Although previous studies have reported the parasitism of buffaloes by *R. microplus*, *A. cajennense*, *A. maculatum*, and *D. nitens* (Miranpuri, 1988), few have evaluated their vector competence for *A. marginale*, especially for *Amblyomma* sp. ticks. However, while *A. marginale* PCR positive ticks were recorded, this does not indicate vector competence; only that the ticks may contain a blood meal from an infected host.

Although *A. marginale* DNA was found in 35 ticks by qPCR, only nine remained positive by conventional PCR for the *msp5* and *msp1α* genes. This might be attributed to the varying sensitivities of PCR protocols despite testing the same sample. Phylogenetic analysis using *msp5* sequences showed that the *A. marginale* detected in all five *R. microplus* ticks and buffaloes were phylogenetically related and that two *A. marginale* isolates from *A. cajennense* (1 and 3) and one from *D. nitens* are separate from the rest of *A. marginale* strains isolated from cattle, buffaloes, and other tick species (Fig. 3), suggesting genetic diversification. However, these ticks were collected from buffaloes in farms in both the southeast and the north of Brazil. Thus, the phylogenetic distance of *A. marginale* strains may also be a result of the geographic distance between the animals studied. Future studies should evaluate the presence of these *A. marginale* isolates in wild animals. The proximity between samples of ticks and the samples from the two buffaloes under study suggests that the isolates present in ticks and in the blood of buffaloes are the same.

In summary, our results suggest that ticks of the species *R. microplus*, *A. cajennense*, *A. maculatum*, and *D. nitens* may be involved in the transmission of *A. marginale* in buffaloes. These results confirm that the same *A. marginale* isolate that circulates in cattle also circulates in buffaloes in Brazil. However, an *A. marginale* isolate found in ticks of the genus *Amblyomma* appears to circulate specifically in buffaloes. The distinction between *A. marginale* isolates in *R. microplus* and *Amblyomma* sp. suggests that different genotypes circulating in the herd may exhibit vector preference.

Conflict of interest statement

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

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