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Original article

Molecular and serological prevalence of *Anaplasma marginale* in water buffaloes in northern Brazil

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ABSTRACT

Bovine anaplasmosis, caused by *Anaplasma marginale*, occurs in tropical and subtropical regions throughout the world and is a major constraint on cattle production in many countries. Approximately 60% of the buffalo herds in South America are located in northern Brazil. However, compared with the research on cattle, research on buffaloes has been neglected. Therefore, the present study was conducted to investigate the distribution of *A. marginale* in water buffaloes in northern Brazil. A total of 500 buffalo blood samples was randomly collected from 16 provinces and was analyzed using both nPCR assay and ELISA techniques. The percentage of animals that were seropositive for *A. marginale* according to ELISA was 49% (245/500). The main risk factors associated with seroprevalence were the region ($p = 0.021$; OR = 1.2) and the reproductive status ($p = 0.0001$; OR = 1.6). *Anaplasma marginale* DNA was detected in 5.4% (27/500) of the sampled buffaloes. Our data provide information about the incidence of *A. marginale* infection in water buffaloes and may guide future programs aimed at controlling the disease in the northern region of Brazil. Although these water buffaloes are exposed to *A. marginale*, a low rate of *A. marginale* PCR-positive animals was found, which could be explained by the habitat in which the sampled animals live because they exhibited a low rate of attached ticks on their skin.

Introduction

Anaplasma marginale, a tick-borne obligate intracellular α proteobacterium belonging to the order Rickettsiales, family Anaplasmataceae, is the agent of bovine anaplasmosis (Dumler et al., 2001). *Anaplasma marginale* is the most prevalent tick-borne pathogen of cattle worldwide, with an endemic occurrence in several regions of the Americas, Africa, Asia, and Australia (Kocan et al., 2010).

Brazil is a developing agricultural country located in South America, where the development of the livestock industry has been hampered by the high occurrence of tick-borne diseases, particularly bovine anaplasmosis (Vidotto et al., 1998). Water buffaloes are located predominantly in the northern part of the country, where they meet many important human needs by providing meat, milk, and leather, plowing the land and transporting people and crops (IBGE, 2012).

Although water buffaloes are raised with cattle and are potential carriers of *Anaplasma*, only Corrêa (2011) and Silva et al. (2013) have reported the occurrence of *A. marginale* among the water buffaloes in Brazil. Therefore, epidemiological surveys of *A. marginale* infections in water buffaloes are expected to be beneficial in reducing the economic losses of the Brazilian livestock industry. For this reason, we investigated the molecular and serological prevalence of *A. marginale* infection in water buffaloes in the northern region of Brazil.

Materials and methods

Design and population studies

A cross-sectional serological and molecular study was conducted from January to December 2011 in buffalo herds in

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16 provinces located in the state of Pará in the northeastern region of Brazil.

We randomly selected a total of 7 provinces on Marajó Island (Soure, Salvaterra, Muaná, Chaves, Ponta de Pedras, Cachoeira do Arari, and Santa Cruz do Arari) and 9 provinces on the continent (Abaetetuba, Ipixuna do Pará, Marapanim, Moju, Nova Timboteua, Tailândia, Paragominas, Santa Izabel do Pará, and São Caetano de Odivelas).

On the continent, the vegetation is predominantly provided by the Amazon rainforest (tropical rainforest). The buffaloes are raised on land and are managed predominantly with beef cattle (Nelore). The buffaloes are vaccinated against brucellosis and foot-and-mouth disease, and they periodically receive endectocidal drugs (ivermectin). Furthermore, these animals are handled in pens using a production system that aims to profit by marketing them. In contrast, large areas of bog and grassland along the floodplains of rivers are found on Marajó Island. In this area, buffaloes are reared in the wetlands. Although they are vaccinated against the same agents as those reared on the continent, endo- and ectoparasite control is rarely used. These animals are reared using an extensive subsistence system.

Sampling

The number of samples required to assess the prevalence of *A. marginale* in the water buffaloes in the northern region of Brazil was determined using the formula recommended by the Pan American Zoonosis Center (CEPANZO, 1979) for the study of chronic diseases, as follows:

$$N = \frac{p(100-p)Z^2}{(dp/100)^2}$$

where N is the number of samples; p is the expected prevalence; Z is the confidence level, and d is the error margin. The estimated prevalence of *A. marginale*-infected buffaloes was 40%. A confidence level of 95.0% and an error margin of 5.0% were established. We estimated that 500 samples should be analyzed. Thus, we randomly selected all of the female water buffaloes on at least 3 farms to provinces, which included 260 buffaloes from Marajó Island and 240 buffaloes from the continent, with 255 belonging to the Murrah breed and 245 to the Mediterranean breed. Regarding their reproductive status, 252 females were pregnant and 248 were not.

Whole blood samples were collected from the caudal or jugular veins of individual water buffaloes. To prepare the serum samples, blood samples collected without the anticoagulant EDTA were incubated at room temperature for 1 h and then centrifuged at 1000 × *g*; then, the sera were collected and stored at −20 °C until use. EDTA-containing blood samples were also stored at −20 °C until they were used for DNA extraction for the PCR assays.

Enzyme-linked immunosorbent assay (ELISA)

An *Anaplasma marginale* isolate from a calf in Jaboticabal in the state of São Paulo, Brazil, generously supplied by Professor Rosangela Zacarias Machado (Unesp-Jaboticabal), was used to infect a calf for crude ELISA and IFAT antigen production. For this purpose, a 3-month-old splenectomized calf was inoculated with 200 mL of *A. marginale*-infected blood (10^7 infected erythrocytes/mL). The rickettsemia peak (10^7 *A. marginale*-infected erythrocytes/mL) was observed 7 days after the experimental infection. After the blood was collected and processed for crude ELISA antigen production (Machado et al., 1997), the experimentally infected animal was treated with oxytetracycline (200 mg/kg).

Briefly, 100 μl of antigen diluted in 0.05 M carbonate/bicarbonate buffer, pH 9.6, was added to each well of a micro-ELISA plate (Immulon; Dynatech Laboratories Inc., Alexandria, VA), and the protein concentration was adjusted to 10 μg/mL. The plates were sealed and incubated overnight at 4 °C. The plates were blocked with 3% ovalbumin in carbonate/bicarbonate buffer for l h at 37 °C in a humid chamber. After 5 washes with PBS-Tween (phosphate-buffered saline, pH 7.2, containing 0.05% Tween 20), 100 μl of diluted bovine sera (1:400) in PBS-Tween plus 5% normal rabbit serum was added in duplicate to the ELISA plate. The plates were incubated at 37 °C in a humid chamber for 90 min and then washed 5 times with PBS-Tween. A 100-μl aliquot of a 1:10,000 dilution of alkaline phosphatase-conjugated anti-bovine IgG (Sigma Chemical Co.) was added to each well, and the plates were incubated at 37 °C under the same conditions for 90 min. The plates were washed 5 times with PBS-Tween. The appropriate substrate (p-nitrophenyl phosphate) was added, and the plates were sealed and incubated for 40 min at room temperature. Finally, the absorbance at a wavelength of 405 nm was read using a micro-ELISA reader (B.T.-100; Embrabio, São Paulo, Brazil). The cut-off values were calculated using receiver operating characteristic (ROC) analysis of the values for 30 non-*Anaplasma*-infected water buffaloes' sera, using MedCalc statistical software (Version 11.4; http://www.medcalc.be) (Terkawi et al., 2011). Thirty sera samples obtained from foals before they suckled colostrum were used as negative controls. A positive reference group consisting of serum samples from 30 buffaloes that tested positive for *A. marginale* (titers of 1280 by IFAT) were used as positive controls for the serological assays.

Semi-nested PCR

DNA was extracted from 200 μL of each of the 500 EDTA whole-blood samples using the QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Initially, the presence of *A. marginale* DNA was detected using 1.0 μl each (20 pmol) of primers targeting part of the *MSP-5* gene, namely Amar msp-5 eF (5′ GCA TAG CCT CCG CGT CTT TC 3′) and Amar msp5 eR (5′ TCC TCG CCT TGG CCC TCA GA 3′), previously described by de Echaide et al. (1998) and optimized by Singh et al. (2012). The PCR protocol was at 94 °C for 5 min, followed by 34 repetitive cycles of 94 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min (Singh et al., 2012). Each sample of extracted DNA was used as a template in 25-μL reaction mixtures containing 10× PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM deoxynucleotide triphosphate (dNTPs) mixture, and 1.0 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The amplified products of the first reaction were used for another PCR using the primers Amar msp5 eR (5′ TCC TCG CCT TGG CCC TCA GA 3′) and Amar msp5 iF (5′ TAC ACG TGC CCT ACC GAG TTA 3′) (Singh et al., 2012). The cycling conditions were the same as for the PCR using the Amar msp-5 eF and Amar msp5 eR primers. Ultra-pure sterile water was used as the negative control. To prevent PCR contamination, the DNA extraction, reaction setup, PCR amplification, and electrophoresis were performed in separate rooms.

Statistical analysis

An individual animal served as the unit of analysis. The serological and molecular results (positive or negative) were the response variables for *A. marginale* in this study. Crude odds ratios (OR) were calculated for each risk factor. The exposure variables were the locations (island and continent), provinces (the 16 provinces studied), breed (Murrah or Mediterranean), and reproductive status (pregnant or nonpregnant).

The confidence intervals of the overall and stratified individual buffalo's seroprevalence for each variable evaluated were calculated as the proportion of ELISA-positive animals divided by the