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Serological and molecular detection of *Theileria equi* in sport horses of northeastern Brazil

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ABSTRACT

Theileriosis is a worldwide protozoal tick-borne disease caused by *Theileria equi*, which may produce a variety of clinical signs and turn infected horses into lifetime carriers. This study has aimed to perform a serological and molecular detection of *T. equi* and associated factors in sports horses from six areas of northeastern Brazil. In overall, 59.6% horses were positive by indirect immunofluorescence assay and 50.4% by polymerase chain reaction. No significant association was found when presence of ticks, age, gender, anemia or total plasma proteins was analyzed with seropositivity and molecular techniques. Although a significant association of infection was found in two cities. Thus, local risk factors other than presence of ticks, horse age, gender, anemia and total plasmatic proteins may dictate prevalence of *T. equi* infection in sports horses, even in highly endemic areas with no control of infection prior to horse competitions.

1. Introduction

Equine theileriosis is a worldwide tick-borne disease caused by the protozoan *Theileria equi* (formerly *Babesia equi*) which infects equids [1–7]. Since severity of clinical signs may vary and horses may present lifetime infection [8], the World Organization for Animal Health has established a serological testing for theileriosis to identify equine carriers [9]. Serological assay limitations on sensitivity and specificity have led to an increase in the use of molecular methods for detection of animal carriers (Box 1) [10–12]. Additionally, molecular approaches may also improve serological testing since equi merozoite antigen 1 (EMA-1), a major surface protein considered the most immunedominant *T. equi* antigen, has been geographically conserved among all known isolates [13].

In Brazil, although *T. equi* has been reportedly transmitted by *Rhipicephalus* (*Boophilus*) *microplus* [14] evidences suggest that infection may be also associated to *Amblyomma cajennense* [15], an intrastadial vector of *T. equi* commonly found in Texas, USA [16]. *Theileria equi* seroprevalence may vary among regions, ranging from 8.2% in Italy [17], 21.6% in Israel [18], 41.6% in Egypt [19], 50.3% in Venezuela [20], to 78.3% in Southern [4] and 81% in Southeastern Brazil [21–24]. However, to the authors' knowledge, no study has been conducted on *T. equi* in horses from northeastern Brazil,

Box 1: Summary of serodiagnostic methods for *Theileria equi*.

Methods	Advantages	Disadvantages
IFA	Ab detection 3–20 days PI	Standardization and subjectivity in the interpretation
CFT	Ab detection 8 days PI	False negative results in latent infection Crossreactivity with *Babesia caballi* Low sensitivity in chronic cases Inconclusive results
cELISA	High specificity Latent infection	False negative in acute infection False positive after clearance of *T. equi*

Ab, antibodies; PI, post-infection.

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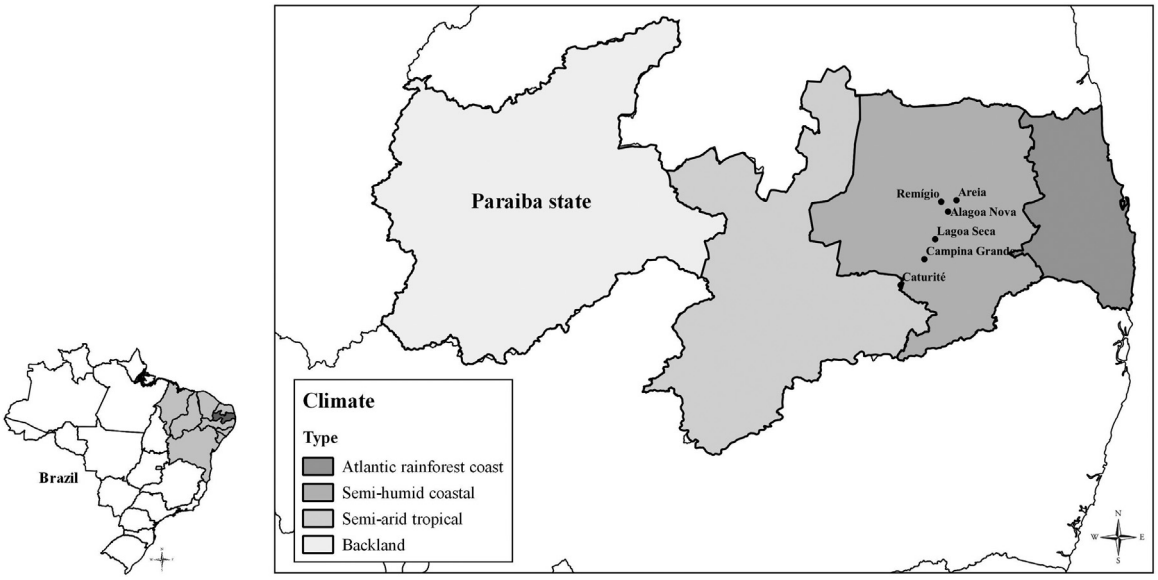

Fig. 1. Geographical locations of horses used in the study and limits of the climate in Paraíba, Northeastern, Brazil.

a tropical climate region where equestrian sports such as "vaquejada" have been routinely practiced [25]. Thus, the present study has aimed to detect *T. equi* and to identify potential risk factors associated with the infection among horses in northeastern Brazil.

2. Materials and methods

This study has been approved by the Ethics Committee in Animal Experimentation and Animal Welfare at the Universidade Federal da Paraíba (protocol 3305/14), Paraíba State, northeastern Brazil.

2.1. Study design

A cross-sectional study approach was employed during the winter from July to August 2013 in five rural and one urban areas in Paraíba State, northeastern Brazil (Fig. 1).

2.2. Samples

A total of 119 blood samples were collected from clinically healthy sports (vaquejada) horses, 72 males and 47 females, of different breeds (65 mixed breed, 47 Quarter Horse, 5 Mangalarga, 1 Appaloosa and 1 Paint Horse) and ages (from 2 to 13 years-old). A total of 97 horses were sampled in rural areas with 20 from Alagoa Nova, 9 from Areia, 6 from Caturité, 16 from Lagoa Seca, and 46 from Remígio (RA); and 22 horses from the urban area (UA) of Campina Grande were used. During the sampling, owners have volunteered answered a questionnaire addressing the horse breed, age, gender and presence of ticks on the horses. Horse ages were later stratified into two groups of <5 years and ≥5 years.

Blood samples (10 mL) were collected by venipuncture of the jugular vein using commercial sterile vacuum tubes containing serum separator gel (BD Vacutainer®, Franklin Lakes, NJ, EUA), and stored at room temperature (25 °C) until visible clot retraction. Samples were then centrifuged at 1500*g* for 5 min, and the serum was separated and stored at −20 °C until serological analysis. Packed cell volume (PCV) and total plasmatic protein (TPP) were performed on EDTA blood samples, as previously described [26]; a PCV of 0.32 L/L or less and a TPP of 80 g/L or more were used as indicators of anemia and hyperproteinemia. Thereafter, aliquots were stored at −20 °C until molecular testing.

2.3. Detection of anti-Theileria equi antibodies

Anti-*T. equi* antibodies in serum samples were evaluated by IFA in 119 samples, as previously described [27]. *Theileria equi* seropositive and seronegative horse serum samples were used as positive and negative controls, respectively. Samples were considered positive when reacting with dilution ≥1:80 [28]. Titers were determined to the largest dilution in which fluorescence was visualized around the parasite (endpoint titers).

2.4. DNA extraction

DNA was extracted from all blood samples using a commercially available kit (Wizard® Genomic DNA Purification, Madison, WI, USA), according to the manufacturer's instructions. Negative control purifications using ultra-pure water were performed in parallel to monitor cross-contamination with each batch of 30 samples.

2.5. PCR assays

A PCR for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed to ensure successful DNA extraction, as previously described [29]. Samples were tested by a conventional PCR for the detection of EMA-1 gene of *T. equi* using a set of previously described primers [30]. Briefly, 3 μL of DNA was used as a template for the amplification, in a total reaction mixture of 25 μL containing 10× PCR buffer (Invitrogen, Carlsbad, CA, USA), 1.5 mM of deoxynucleoside triphosphate (dNTPís), 0.25 U of *Taq*DNA polymerase (Platinum®, Invitrogen, Carlsbad, CA, USA) and 0.1 mM of each primer. After initial denaturation at 94 °C for 2 min, the amplification consisted of 40 cycles of 30 s each at temperatures of 94 °C, 58 °C and 72 °C for denaturation, annealing and extension, respectively, with a final extension at 72 °C for 30 s; the samples were kept at 4 °C until analyzed. The amplified PCR products were subjected to gel electrophoresis in 1.5% agarose gels for one hour at 100 V, followed by SYBR safe staining (6 μg/mL), and were viewed under a 312 nm UV light transilluminator. The gels were subsequently photographed using Kodak DC290 (New York, EUA). Additionally, samples were screened for hemotropic mycoplasmas (hemoplasmas) infection by PCR, as previously described [31].