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Seroprevalence rates of antibodies against *Theileria equi* in team roping horses from central-western region of Paraná

Soroprevalência de anticorpos contra *Theileria equi* em equinos atletas da região centro-oeste do Paraná

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Abstract

The purpose of this study was to estimate the prevalence of *Theileria equi* in horses from central western region of Paraná state, Brazil. The presence of antibodies IgG against *T. equi* was determined in serum samples obtained from 400 team roping horses of the district of Guarapuava by the enzyme-linked immunosorbent assay (ELISA). Results showed that 242 (61%) animals were positive which demonstrates that equine piroplasmosis is widespread and therefore it might be a contributing factor for the irregular performance among athletes horses in the region studied. No association regarding age and sex were observed ($p > 0.05$). To our knowledge, this is the first report describing a serological survey on equine piroplasmosis in the state of Paraná, Brazil.

Keywords: *Theileria equi*, horses, ELISA.

Resumo

O objetivo deste estudo foi estimar a prevalência de *Theileria equi* em equinos da região centro-oeste do Estado do Paraná, Brasil. A presença de anticorpos IgG contra *T. equi* foi determinada em amostras de soro obtidas a partir de 400 cavalos atletas do distrito de Guarapuava pelo ensaio imunoenzimático (ELISA). Os resultados mostraram que 242 (61%) animais foram positivos, o que demonstra que a piroplasmose equina apresenta ampla distribuição e, portanto, poderá contribuir para a performance irregular de cavalos que participam de eventos desportivos na região. Não foi observada associação com a idade ou sexo dos equinos ($p > 0,05$). Pelo que se sabe, este é o primeiro relato de levantamento sorológico sobre piroplasmose equina no Estado do Paraná, Brasil.

Palavras-chaves: *Theileria equi*, horses, ELISA.

Equine piroplasmosis, caused by *Theileria equi* and *Babesia caballi*, has emerged as an important protozoan infection from the veterinary and economic viewpoints. The disease is generally characterized by fever, anemia, icterus, prostration, hepato and splenomegaly, intravascular hemolysis, hemoglobinuria and bilirubinuria and in some cases, can lead to death (SCHEIN, 1988). However, clinical signs are not pathognomonic and may vary from asymptomatic to acute, being most of the times variable and non-specific (DE WAAL, 1992). Indeed, most of the horses act as carriers of the parasite for several years, becoming reservoirs

for vector ticks (KNOWLES et al., 1996; CACCIO et al., 2000). To date, three distinct genera of ticks (*Dermacentor*, *Hyalomma* and *Rhipicephalus*) have been identified in *T. equi* transmission (MEHLHORN; SCHEIN, 1998).

The disease has a worldwide distribution and is endemic in most tropical and subtropical areas as well as in some temperate zones of the world (SCHEIN, 1988; DE WAAL, 1992; BRUNING, 1996). It has caused important economic losses in the horse industry, being a serious threat to the horse raising industry and international movement of horses (FRIEDHOFF et al., 1990). Previous studies have demonstrated losses due to treatment costs, recuperation time and low athletic performance (GARCÍA-SANMARTÍN et al., 2008). In addition, equine piroplasmosis creates difficulty in animals trading and restrictions regarding import/export of animals, as well as participation in international

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competitions, given that infected animals are prevented from entering countries that are disease-free (FRIEDHOFF et al., 1990; KNOWLES, 1996).

In Brazil, *T. equi* is considered endemic due to high levels of tick infestation on horses. Previous studies in southern and central areas of the country have reported occurrences varying from 49.2% to 100% (HEUCHERT et al., 1999; CUNHA et al., 1996; LABRUNA et al., 2001; XUAN et al., 2001; BALDANI et al., 2004, 2010; HEIM et al., 2007).

In the light of reports of irregular performance among athlete horses in the central-western region of Paraná, the aim of the present study was to determine the occurrence of *T. equi* infections by Enzyme-linked Immunosorbent Assay (ELISA) in horses among team roping from the municipality of Guarapuava, state of Paraná, Brazil.

A total of 400 team roping horses from Guarapuava district, central-western of Paraná State (with an south latitude 25° 23' 26", west longitude 51° 27' 15" and altitude of 1,116.5 m) were randomly selected, in which breeds comprised American quarter and Criollo, being 195 female and 205 male, independent of age. For analyses, the factors age (<12 months; 1-5 years; 5-10 years; and >10 years) and gender were considered. The study was carried out between January of 2005 and December of 2007. Serum samples were subjected to an Enzyme-linked Immunosorbent Assay (ELISA) to detect IgG antibodies against *T. equi* as described by Baldani et al. (2004).

ELISA antigen was obtained from a splenectomized horse with the *T. equi* Jaboticabal strain (GenBank accession number: DQ250541). During the peak of parasitaemia ml of blood was collected in an equal volume of Alsever solution, approximately 80% of erythrocytes were infected. The blood was diluted 1:4 in normal saline and the infected erythrocytes subjected to lyses with ammonium chloride (MACHADO et al., 1994). *T. equi* free merozoites were disrupted by nine freezing (-70°C)/thawing cycles (37°C), lyophilized and stored at -70°C until required for the ELISA test.

Ninety-six-well microtitration plates (NuncloTM Surface; Nunc, Denmark) were coated overnight at 4°C with 100 µL (10µg/mL) of crude *T. equi* antigen diluted in a sodium bicarbonate-carbonated 0.05M buffer (pH 9.6). After overnight incubation at 4°C, the excess of antigen was removed by three washes with PBS Tween-80 at 0.05%. To reduce non-specific binding, the plates were blocked with 200 µL of PBS Tween-80 containing 6% skim milk for 2 hours at 37°C. The blocking agent was removed, and individual horse serum diluted 1:100 in PBS Tween-80 with 5% skim milk (PBS-TW-SM) was added to each well and then incubated for 90 minutes at 37°C. Unbound antibodies were removed by washing the plates as described above. One hundred µL of alkaline phosphatase conjugated anti-horse IgG (Sigma Chemical Co.) diluted 1:15000 in PBS-TW-SM were added to each well and then incubated for 90 minutes at 37°C. The plates were washed and the appropriate substrate (p-nitrophenyl phosphate) was added. Absorbance at 405 nm was read after 45 minutes incubation at room temperature using an ELISA reader (Dynex Technologies).

The immunological activity of each serum was calculated by determining the sample to positive serum ratio (S/P), considering positive and negative sera as reference, using the following equation:

(mean sample absorbance - mean absorbance of negative serum reference)/(mean absorbance of positive reference serum - mean absorbance of negative serum reference) (MACHADO et al., 1997). S/P values were grouped into ELISA levels (EL), which ranged from 0 (lowest level) to 9 (highest level). The discriminant absorbance value (cut-off) was determined as being two and a half times the mean absorbance value of the negative group. Horses were considered to be positive when S/P values at an optical density of 405 nm were equal or greater than 0.278 (EL=2).

The *T. equi* positivity in the horses, as determined by ELISA, was tested for association with age and gender using the chi-squared or Fisher's exact tests at a 5% significance level.

Equine piroplasmiasis is an important disease especially for horses that travel to equestrian sporting events. Moreover, economic losses due to low performance associated to the disease should be considered. In the present study, 242 (61%) samples were serological positive for *T. equi*. These results shows that *T. equi* is widespread in the region studied, suggesting high levels of transmission. Additionally, our data is in accordance with previous studies which have demonstrated that the disease is endemic in Brazil (CUNHA et al., 1996; BALDANI et al., 2004; HEIM et al., 2007; SANTOS et al., 2011; PECKLE et al., 2013). To our knowledge, this is the first report of the detection of IgG antibodies anti-*T. equi* in Paraná state.

The variables inherent to equines, such as gender and age, were not associated with *T. equi* positivity ($p>0.05$), as 127 male and 125 female were positive. The lack of association with gender is consistent with other studies (SHKAP et al., 1998; SOUZA et al., 2000; BOTTEON et al., 2002; KOUAM et al., 2010). Considering age, seropositivity was found in 82 animals with 1-5 years; 122 5<10; and 38 ≥10 years. The lack of association between age and *T. equi* is consistent with several reports in the literature (SHKAP et al., 1998; SOUZA et al., 2000; BOTTEON et al., 2002; MORETTI et al., 2010). However, other studies found inconsistent results (RÜEGG et al., 2007; KOUAM et al., 2010), what could be explained in part by different age categorization.

Several authors have demonstrate that the prevalence rates for *T. equi* ranges from 17 to 100%, according to the region studied and to the diagnosis method applied (PFEIFER BARBOSA et al., 1995; HEUCHERT et al., 1999; KERBER et al., 1999, 2009; XUAN et al., 2001). The overall prevalence of 61% of equine piroplasmiasis in the present study is relatively high and is in accordance with other studies performed in the southern region of Brazil. Cunha et al. (1996) in Rio Grande do Sul state reported the occurrence of antibodies anti-*T. equi* by indirect fluorescent antibody test (IFAT) in 57.8% of the horses from Pelotas Jockey Club and two farms. On the plateau of Santa Catarina, Souza et al. (2000) observed that the prevalence of *T. equi* by IFAT was 50.3%.

However, in southeastern Brazil the occurrence of *T. equi* is much higher. In the state of São Paulo, Xuan et al. (2001) and Baldani et al. (2004, 2010) reported a prevalence of 81%, 75% and 100% by ELISA for *T. equi*, respectively. Pfeifer Barbosa et al. (1995), using the IFAT, also observed a prevalence of 100% for *T. equi* in horses of Rio de Janeiro state. Furthermore, Bittencourt et al. (1997) found that the prevalence was 84.6%, using the complement fixation test (CFT) in horses of the same state. Heim et al. (2007) reported seroprevalence of 91.0% by IFAT

for *T. equi* in samples collected from horses at a slaughterhouse in the state of Minas Gerais, southeast Brazil.

The differences observed in the seropositivity of *T. equi* might be a result of different serological tests used. Several studies have showed the low sensitivity and specificity of CFT in identifying equine piroplasmosis carriers, and while they rarely give false positive results, it may occasionally result in a negative response in horses with latent infections (WEILAND, 1986). IFAT, on the other hand, is more sensitive than CFT and rarely renders false negative results (TENTER; FRIEDHOFF, 1986), however standardization is difficult, considering the subjectivity of the reader in assessing the results (BOSE, 1995; BRUNING, 1996). Therefore, ELISA is nowadays an alternative for increased specificity and sensitivity in the detection of acute and latent babesial infections (XUAN et al., 2001; HIRATA et al., 2003; KUMAR et al., 2003; BALDANI et al., 2007). It should be mentioned that *T. equi* antigen used in the present study, was produced using a very simple methodology and required little material manipulation at a very low cost. Additionally, a previous study have demonstrated that an ELISA with crude soluble antigen was 100% sensitive and specific (BALDANI et al., 2004). Also important, in the present study the antigens was obtained from a Brazilian *T. equi* strains, which eliminates possible antigenic differences existent between isolates from different regions (KUTTLER et al., 1988).

Another possible explanation for the differences in the seroprevalence rates of *T. equi* might be related to the restriction of the living area of the horses, especially considering presence of tick vectors. In one previous report (BALDANI et al., 2010) the high positivity rate for *T. equi* was attributed to the management of horses, which appears to be an important factor for the prevalence of *T. equi* infections. Additionally, climatic factors such as temperature, relative humidity, altitude and high rainfall influence the habitat of the main tick/vector species that parasite horses (GOLYNSKI et al., 2008). It has been demonstrated that when horses have direct or indirect contact with cattle and there is no rigorous tick control program, *T. equi* infection rates are much higher (HEUCHERT et al., 1999; KERBER et al., 1999).

In Latin America, horses are regularly infested with three species of ticks: *Dermacentor nitens*, *Rhipicephalus (Boophilus) microplus*, and *Amblyomma cajennense* (BORGES; LEITE, 1998; LABRUNA et al., 2001; DA COSTA PEREIRA et al., 2005). *R. (B.) microplus* has been implicated as a vector of *T. equi* (GUIMARÃES et al., 1998; UETI et al., 2008), while the role of *D. nitens* in its transmission has not been reported (DENNING, 1988). As so, the lower prevalence observed in the region of the present study compared to southeastern regions of Brazil may be due to the conditions encountered by tick vectors which are less favorable for their development and reproduction, contributing therefore for lower tick infestation (DAVEY; COOKSEY 1989; CHACÓN et al., 2003). It should be mentioned that the municipality of Guarapuava, which is located in the high-altitude region of the state of Paraná, has a humid semitropical climate, with an annual average temperature of approximately 16 °C, with winter temperatures that reach bellow 0 °C. Peckle et al. (2013) also reported lower infection frequency of *T. equi* in horses of Petrópolis, a region of Rio de Janeiro state with high altitude,

where the annual average temperature is 16 °C and winter temperatures of 0 °C.

In conclusion, this study reports the occurrence of antibody seroreactivity against *T. equi* in team hoping horses of Guarapuava, central-western region of Paraná state. Accurate diagnosis of equine piroplasmosis is essential for providing baseline information about its epidemiology, distribution and prevalence in the affected equine population, and is thus a prerequisite for elaborating appropriate and effective control measures. The overall *T. equi* seroprevalence of 61% showed here could be related to irregular performance among athletes horses.

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