

# Occurrence of *Theileria equi* in horses raised in the Jaboticabal microregion, São Paulo State, Brazil

Ocorrência de *Theileria equi* em equinos criados na microrregião de Jaboticabal, Estado de São Paulo, Brasil

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## Abstract

Blood and serum samples from 170 horses raised in the Jaboticabal microregion, São Paulo State, Brazil, were collected and tested by microscopic examination of blood smears, indirect fluorescent antibody test (IFAT) and nested polymerase chain reaction (nPCR) for *Theileria equi* infections. The association among the test results was verified by the McNemar test. During the examination of thin blood smears, parasites were detected in six (3.52%) horses. Anti-*T. equi* antibodies were detected in 100% sera samples, with titers ranging between 1:80 and 1:5120. The nPCR based on the *T. equi* merozoite antigen gene (*EMA-1*) allowed the visualization of specie-specific amplified product in 108 (63.53%) horses. All six samples judged positive microscopically were also positive for nPCR. Statistical analysis indicated general disagreement ( $p < 0.0001$ ) between IFAT and nPCR; IFAT and blood smear; and nPCR and blood smear on the detection of parasite carriers. The results of the present study indicate that *T. equi* is widely spread among horses in the Jaboticabal microregion, Northeast region of São Paulo State, Brazil.

**Keywords:** *Theileria equi*, horse, diagnosis, IFAT, nested PCR.

## Resumo

Amostras de sangue e soro de 170 equinos criados na microrregião de Jaboticabal, Estado de São Paulo, Brasil, foram coletadas e avaliadas pelo exame direto em esfregaço sanguíneo, reação de imunofluorescência indireta (RIFI) e *nested* reação em cadeia da polimerase (nPCR) para a detecção de infecções por *Theileria equi*. A concordância dos resultados entre os testes de diagnóstico foi verificada pelo teste de McNemar. Durante o exame dos esfregaços sanguíneos, parasitos foram detectados em seis (3,52%) equinos. Anticorpos anti-*T. equi* foram detectados em 100% das amostras de soros, com títulos variando entre 1:80 e 1:5120. O nPCR, baseado na sequência do gene do antígeno de merozoíto de *T. equi* (*EMA-1*), permitiu a visualização de produtos de amplificação espécie-específico em 108 (63,53%) equinos. Houve diferença altamente significativa ( $p < 0,0001$ ) entre RIFI e nPCR; RIFI e esfregaço sanguíneo; e nPCR e esfregaço na detecção do parasito. O resultado do presente estudo indica que a infecção por *T. equi* está amplamente distribuída entre os equinos na microrregião de Jaboticabal, região Nordeste do Estado de São Paulo, Brasil.

**Palavras-chave:** *Theileria equi*, equino, diagnóstico, RIFI, nested PCR.

## Introduction

*Theileria equi* is a protozoan of the phylum Apicomplexa that is biologically transmitted by ixodid ticks. It causes disease in equids characterized by fever, anemia, icterus, hepatosplenomegaly, intravascular hemolysis, hemoglobinuria and in some cases death can occur (SCHEIN, 1988). However, most of the animals that

recover from an acute or primary infection are carriers of the parasite for several years, becoming reservoirs for vector ticks (CACCIÒ et al., 2000; KNOWLES, 1996). Additionally, *T. equi* infection can be suppressed by chemotherapy but it cannot be completely eliminated (DE WAAL; VAN HEERDEN, 1994).

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 (BRÜNING, 1996; DE WAAL, 1992; SCHEIN, 1988). 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).

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Indeed, there are regulatory import restrictions in some disease-free countries such as United States, Canada, Japan, Australia and New Zealand (BRÜNING, 1996). Nowadays, regulations usually require serological testing of horses in order to confirm seronegativity and to identify seropositive animals whose movement is restricted (BÖSE et al., 1995; BRÜNING, 1996).

Equine piroplasmiasis can be diagnosed by several different methods. Direct microscopic identification of the parasite in stained blood smears is confirmatory, but it is usually difficult to find the organism in carrier animals since parasites are generally present in very low numbers in the blood. Therefore, despite its high specificity, microscopic examination of blood smears has low sensitivity for the detection of carrier animals (BÖSE et al., 1995). Hence several serological tests have been developed and are of great interest for the detection of specific antibodies, such as complement fixation test (CFT), indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assays (ELISA). The World Organization for Animal Health (2008) currently recommends IFAT or ELISA as tests of choice for the diagnosis of *T. equi* infection. Several studies have showed low sensitivity and specificity of CFT in identifying equine piroplasmiasis carriers, and while they rarely give false positive results, it may occasionally result in a negative response in horses with latent infections (WEILAND et al., 1986). IFAT, on the other hand, is commonly used to detect specific antibodies to *T. equi*, especially in cases where CFT has proven to be inconclusive (BRÜNING, 1996). Although IFAT is more sensitive than CFT and rarely renders false negative results (TENTER; FRIEDHOFF, 1986), standardization is difficult, considering the subjectivity of the reader in assessing the results (BÖSE et al., 1995; BRÜNING, 1996). ELISA is nowadays an alternative for increased specificity and sensitivity in the detection of acute and latent babesial infections and several procedures of ELISA using recombinant antigen or crude antigen have been standardized (BALDANI et al., 2007; KUMAR et al., 2003; HIRATA et al., 2003; XUAN et al., 2001; KATZ et al., 2000).

Moreover, molecular diagnostic tools such as polymerase chain reaction (PCR) have been proven useful for the detection of equine piroplasmiasis. The assays are mainly based on ribosomal 18S RNA sequence or *EMA-1* gene and have showed to have high sensitivity and specificity (BALDANI et al., 2008; CACCIÒ et al., 2000; FRITZ, 2010; HEIM et al., 2007; NICOLAIEWSKY et al., 2001; RAMPERSAD et al., 2003). Therefore, this direct diagnostic method has now been incorporated to routine diagnosis, especially in association with serological assays. Another diagnostic technique available for equine piroplasmiasis is *in vitro* blood culture, which has been used for the detection of carrier host animals. Although considered reliable and species-specific, it is a very time-consuming technique and unsuitable for routine diagnosis (BALDANI et al., 2008; HOLMAN et al., 1997). Therefore, the advantages and disadvantages of each test should be considered while choosing the best test for the diagnosis of equine piroplasmiasis.

The objective of this study was to apply IFAT, nPCR and direct microscopic examination to determine the occurrence of *T. equi* infections in horses from stud farms in the Jaboticabal microregion, São Paulo State, Brazil, as well as to compare the performance of these diagnostic techniques.

## Material and Methods

### 1. Horses and sample collection

A total of 170 horses were tested for *T. equi*, which were randomly selected from 8 stud farms of variable size in the Jaboticabal microregion (21° 15' 17" S and 48° 19' 20" W), Northeast region of São Paulo State, Brazil. The region is mainly hilly and is 0-605 m above sea level (INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA, 2007). The average annual temperature is 22 °C. According to the Koppen classification, the climate is of Cwa type, i.e., mesothermic with hot and rainy summers and dry winters. The study was carried out between October 2003 and June 2004. Horse owners participated voluntarily and signed a consent form which clarified the objective of the study. At the moment of sample collection no clinical signs characteristic of equine babesiosis were seen and all owners stated that despite a tick control program in their farms, horses had been exposed to tick infestation.

Blood samples were collected from jugular venipuncture and placed in sterile tubes with and without anticoagulant EDTA. Serum samples were obtained by centrifugation at room temperature (25 °C) at 3000 rpm for 10 minutes and were stored at -20 °C until serological analysis. Tubes containing EDTA were either used immediately for blood smears or stored at -20 °C for later use in nPCR.

### 2. Blood smears

Thin blood smears were prepared from anticoagulated blood, stained with Giemsa and then examined under light microscopy (magnification 1000×; 100 fields) to check for the presence of intracellular parasites.

### 3. Indirect fluorescent antibody test (IFAT)

IFAT antigen was prepared from blood of horses experimentally infected with Jaboticabal *T. equi* strain (GenBank accession no. AF255730) and its sensitivity and specificity were assessed in a previous study (BALDANI et al., 2007). IFAT procedure essentially followed those described by Baldani et al. (2007). Sera were considered positive if they showed strong fluorescence of parasites at a dilution of 1:80 or above. The size, appearance and density of staining were compared with a positive control in which a positive reaction was seen as apple green fluorescent inclusions within infected erythrocytes while a negative reaction showed a uniform red counter stain.

### 4. Nested PCR (nPCR)

DNA extraction was performed from EDTA-whole blood sample by the Puregene Kit (Gentra Systems Inc., USA) in accordance with the manufacturer's protocol. DNA concentrations were determined using the NanoDrop ND-1000 spectrometer (NanoDrop Technologies, DE, USA). Nested PCR assays

were performed as described previously by Nicolaiewsky et al. (2001) and Baldani et al. (2008). The primers sets EMAE-F (5'-CCGCCCTTCACCTCGTTCTCAA-3')/EMAE-R (5'-TCTCGGCGGCATCCTTGACCTC-3') and EMAI-F (5'-CCGTCTCCGTTGACTTGGCCG-3')/EMAI-R (5'-GGACGCGCTTGCCTGGAGCCT-3') were chosen to flank 396 and 102 bp regions of the *EMA-1* gene sequence (GenBank accession number L13784), respectively. PCR products were detected by electrophoresis on 1% agarose gel stained with ethidium bromide.

### 5. Statistical analysis

Statistical analysis was performed using the McNemar test in which the association between direct microscopic identification of the parasite in stained blood smears, IFAT and nPCR were assessed comparing positive rates obtained for *T. equi* diagnosis.

## Results

Of 170 blood samples examined by microscopic observation of Giemsa-stained blood smears, merozoites of *T. equi* were detected in six horses (3.52%). Intraerythrocytic stages of *T. equi* appeared typical and care was taken to differentiate *B. caballi* from *T. equi* in smears of horses.

The overall antibody frequencies at titers 1:80 or greater for *T. equi* in 170 horses from the stud farms studied are showed in Figure 1. Anti-*T. equi* antibodies were detected in all 170 (100%) horses, with antibody titers ranging between 1:80 and 1:5120. The highest frequencies of titers were 1:1280 and 1:2560 in 52 (30.58%) and 39 (22.94%) horses, respectively.

The specific *T. equi* merozoite 102 pb gene fragment (*EMA-1*) was detected in 108 (63.53%) horses. The results of nPCR in horse blood samples are showed in Figure 2. All 6 samples judged positive microscopically were also positive for nPCR with *T. equi* specific primers. However, 102 horses found to be negative by microscopic examination were positive by nPCR.

Statistical analysis showed differences ( $p < 0.0001$ ) between IFAT and nPCR; IFAT and blood smear; and nPCR and blood smear in the diagnosis of *T. equi* infection.

## Discussion

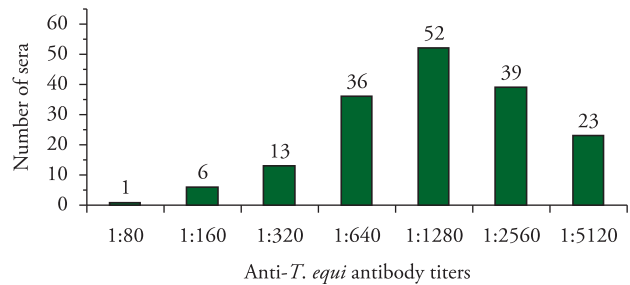
Equine piroplasmosis is of great importance due to the international movement of horses, especially for horses that travel to equestrian sport events. And because of the upcoming V Military World Games in 2011 and the 2016 Olympic Games, both in the city of Rio de Janeiro, there is an increased concern in Brazil due to risk of transmission of the parasite from carrier horses (FRIEDHOFF, 1988). Moreover, some countries maintain stringent restrictions that prevent import of horses serologically positive for piroplasma species (BRÜNING, 1996).

In the present study, the occurrence of *T. equi* infection in horses of the Jaboticabal microregion was investigated by blood smear identification, IFAT and nPCR. The results demonstrate

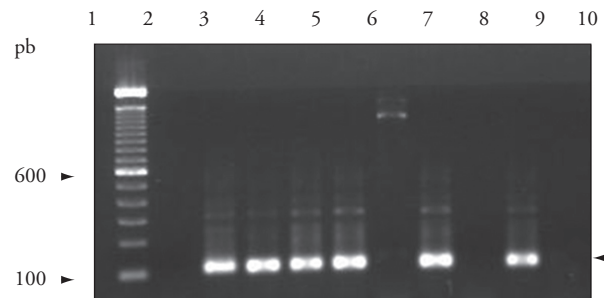
that *T. equi* is widespread in the region studied, suggesting high levels of transmission as there was found a high rate of positive animals by serological and molecular methods. Additionally, high titers of specific antibodies were detected in the IFAT test.

The positive rate of *T. equi* seen in the present study on direct microscopic identification was very low and is consistent with previous studies (RAMBERSAD et al., 2003; HEIM et al., 2007; SEVINC et al., 2008; MORETTI et al., 2010). Despite its high specificity, this method lacks sensitivity, especially in the diagnosis of subclinical infections when parasitemia becomes too low to detect positive cases (BÖSE et al., 1995). Statistical analysis showed significant differences ( $p < 0.0001$ ) between IFAT and blood smear in the diagnosis of *T. equi* infection. All horses in the present study were adult and had been exposed to ticks and consequently to the parasite several times as demonstrated by IFAT results in which all horses were seropositive. As so the majority probably developed protective immunity and became chronically infected carriers, explaining the results found in the study. Similarly, the horse population studied did not have clinical signs of piroplasmosis.

In our study, nPCR revealed that 108 (63.53%) horses were positive for *T. equi*, and all were positive by serology (108/108). All



**Figure 1.** Frequency of IFAT IgG antibody titers against *T. equi* in naturally infected horses (n = 170) raised in the Jaboticabal microregion, São Paulo State, Brazil.



**Figure 2.** Nested PCR (nPCR) of *T. equi* DNA isolated from blood of naturally infected horses raised in the Jaboticabal microregion, São Paulo State, Brazil. Band 1, 100 pb ladder DNA marker (Invitrogen); band 2, DNA from uninfected horses (negative control); band 3, nPCR with DNA of an infected horse (Jaboticabal strain, Genbank DQ250541) (positive control); bands 4 to 11, nPCR with DNA from eight different horses. The amplified samples were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light.



six samples positive microscopically were also positive for nPCR. Molecular methods have been extensively used to detect *T. equi* infection (CACCIÒ et al., 2000; NICOLAIEWSKY et al., 2001; RAMPERSAD et al., 2003; HEIM et al., 2007; BALDANI et al., 2008; FRITZ, 2010). The nPCR using oligonucleotides designed on the sequence of a *T. equi* merozoite antigen gene (*EMA-1*) used in the present study was able to detect the parasite in blood with a corresponding parasitemia of 0.000008% (BALDANI et al., 2008) or 0.000006%, equal to six infected cells out of  $10^8$  erythrocytes (NICOLAIEWSKY et al., 2001). As expected, significant differences ( $p < 0.0001$ ) were detected between nPCR and blood smear for the detection of parasite carriers. The enhanced sensitivity of this direct diagnosis method contributes for high prevalent rate of *T. equi* in blood samples and is comparable to those reported in several other studies conducted in South Africa (BHOORA et al., 2009) and France (FRITZ, 2010), although with different set of primers or using real-time PCR. In Brazil, Heim et al. (2007) evaluated horses from different endemic regions and reported a prevalence of 59.7% for *T. equi* by a multiplex real-time PCR. And Battsetseg et al. (2002) evaluated blood samples from horses of Mato Grosso state and found 96% of positive rate for *T. equi* by nPCR.

Moreover, 62 (36.47%) horses were positive only by IFAT, being negative by both direct diagnosis methods, nPCR and stained blood smears. As so significant differences ( $p < 0.0001$ ) between the three tests were detected. Antibodies can still be found after the parasite has been eliminated (JAFFER et al., 2010), but *T. equi* parasites are usually not completely eliminated from blood of horses after treatment or natural recovery, and horses may remain as lifelong carriers (DE WAAL, 1992). Additionally, molecular detection of these agents requires DNA isolation from parasites that are physically present in the blood sample to a detectable level above the sensitivity threshold of the method used (SALIM et al., 2008). Therefore, failure to detect *T. equi* by nPCR is most probably due to parasite clearance from the circulating blood or reduction to a level beyond the detection sensitivity of the assay used.

The study of equine piroplasmiasis in Brazilian horses showed that the disease is a serious problem in the country and it is considered to be endemic. Previous epidemiological studies carried out in several Brazilian states show prevalence rates ranging from 17 to 100% (PFEIFER BARBOSA et al., 1995; HEUCHERT et al., 1999; KERBER et al., 1999; XUAN et al., 2001). Our results showed high prevalence for *T. equi* in the northeast region of São Paulo, and although relatively higher, they are consistent with those reported by Xuan et al. (2001) and Baldani et al. (2004) in the state, with a prevalence of 81 and 75% for *T. equi*, respectively. Heim et al. (2007) reported seroprevalence of 91.0% for *T. equi* in samples collected from horses at a slaughterhouse in the state of Minas Gerais, Southeast Brazil, in which some horses were originally from São Paulo State. However, Heuchert et al. (1999) tested 752 serum samples from the state of São Paulo by IFAT and CFT and reported prevalence rates of 29.6 and 17.6%, respectively. More recently, Kerber et al. (2009) reported an overall prevalence of 21.6% for *T. equi* by CFT and eELISA in horses of stud farms also in the State of São Paulo. The rate of equine piroplasmiasis found in this study is significantly higher and is probably due to differences in the management of horses, which appears to be an

important factor for the prevalence of *T. equi* infections, and, to a lesser extent, due to the number of serum samples examined. 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). Indeed, the horses studied had history of contact with cattle and although there was tick control, several of them had tick infestations. The detection of high titers in more than half of the horses suggests that these animals are constantly exposed to ticks infected by *T. equi*. Considering that *T. equi* may remain as a lifelong infection (DE WAAL, 1992), it is likely that the horses of the present study are carriers, transmitting the parasite to ticks.

It has been pointed out that serological and molecular assays can be a more objective tool for the diagnosis of equine piroplasmiasis (PERSING; CONRAD, 1995). Thus, it can be reasonably concluded that *T. equi* infections are highly prevalent in the Jaboticabal microregion, Northeast region of São Paulo State, and therefore the establishment of an appropriate and effective control program is of great importance. Despite the healthy appearance of horses in the present study, these carriers can transmit the parasite to ticks and are a potential continuous source for maintaining and disseminating the organism in horse population. Given the territorial extension of Brazil, further investigations on equine piroplasmiasis are required for providing baseline information about its epidemiology, distribution and tick vectors.

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