In vitro culture, PCR , and nested PCR for the detection of *Theileria equi* in horses submitted to exercise

[*Cultivo* in vitro*, PCR e nested PCR na detecção de* Theileria equi *em eqüinos submetidos a exercícios*]

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ABSTRACT

This study compared the usefulness of *in vitro* culture, PCR, and nested PCR for the diagnosis of *Theileria equi* in horses submitted to stress during exercise. Blood samples from 15 apparently healthy horses, previously conditioned to a high-speed equine treadmill, were taken prior to and after exercise. The animals were divided into two experimental groups: 30-day training schedule (G1) and 90-day training schedule (G2). Statistical analysis was performed using a chi-square test and *kappa* statistic was used in order to assess agreement. No significant difference was observed between samples collected at resting or after exercise. In G1, merozoites of *T. equi* were detected in the blood smears of four horses before *in vitro* culture, whereas 14 samples were positive, confirmed by culture. In G2, five and 11 horses were positive before and after culture, respectively. No PCR amplified product was observed in any of the tested animals although the PCR system based on the 16S rRNA gene of *T. equi* detected DNA in blood with an equivalent 8×10^{-5}% parasitaemia. The nested PCR based on the *T. equi* merozoite antigen gene (*EMA-1*) allowed the visualization of amplified products in all the horses. Therefore, nested PCR should be considered as a means of detection of sub-clinical *T. equi* infections and *in vitro* culture could be used as a complement to other methods of diagnosis.

Keywords: horse, *Theileria equi,* diagnosis, *in vitro* culture, PCR

RESUMO

Comparou-se a utilização do cultivo in vitro, PCR e nested PCR no diagnóstico de Theileria equi *em eqüinos submetidos ao estresse induzido por exercícios. Amostras de sangue foram obtidas de 15 eqüinos submetidos a treinamento em esteira rolante de alto desempenho, sendo as amostras colhidas antes e após os exercícios. Os animais foram divididos em dois grupos experimentais: 30 dias de treinamento (G1) e 90 dias de treinamento (G2). O teste do qui-quadrado foi empregado para as análises estatísticas e o índice kappa utilizado para avaliar a concordância. Não houve diferença significativa entre as amostras obtidas em repouso e após exercícios. Merozoítas de T. equi foram detectados em apenas quatro eqüinos do G1 pela microscopia direta realizada antes do cultivo, enquanto 14 animais apresentaram-se positivos pelo cultivo in vitro. No G2, cinco e 11 eqüinos foram positivos antes e após o cultivo, respectivamente. Nenhum produto de amplificação foi observado pela técnica de PCR, apesar do PCR baseado no gene 16S rRNA de* T. equi *ter detectado DNA em sangue com parasitemia equivalente a 8×10^{-5}%. O nested PCR, baseado na seqüência do gene do antígeno de merozoíta de* T. equi *(EMA-1,) permitiu a visualização de produtos de amplificação em todos os eqüinos testados. O nested PCR deve ser considerado para a detecção de infecções subclínicas de* T. equi *e o cultivo in vitro pode ser utilizado como uma ferramenta complementar a outros métodos de diagnóstico.*

Palavras-chave: eqüino, *Theileria equi,* diagnóstico, cultivo *in vitro,* PCR

INTRODUCTION

Theileria equi is a tick-transmitted protozoan parasite and one of the causative organisms of equine piroplasmosis. The disease is characterized by fever, anemia, icterus, hepatomegaly, splenomegaly, intravascular hemolysis, petechial hemorrhages of the mucuous surfaces; hemoglobinuria, and death can occur in some cases (Schein, 1988). Equine piroplasmosis has worldwide distribution, being endemic in most tropical and subtropical areas of the world as well as in some temperate climatic zones (Schein, 1988; De Waal, 1992; Bruning, 1996). Low level carriers animals or ticks, which may act as reservoirs, represent a risk of these parasites being introduced to disease-free areas such as Japan, Australia, and North America (except in the state of Florida, USA). Therefore, the detection of sub-clinical infections has become very important, with special relevance to the horse-racing industry in which the geographical movement of presumably healthy horses may aid the spread of *T. equi* or where sub-clinical infection may negatively affect the performance of the animal (Rampersad et al., 2003). It has been shown that strenuous exercise, such as that experienced with horse-racing or endurance, may predispose clinical manifestation of the disease (Hailat et al., 1997).

Equine piroplasmosis can be diagnosed by means of several 'methods. Direct microscopic identification of the parasite in stained blood smears can confirm diagnosis, 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 the high specificity, microscopic examination of blood smears has low sensitivity for the detection of carrier animals. Serological methods, such as complement fixation test (CFT) and the indirect fluorescent antibody test (IFAT), are commonly used for detecting *T. equi* infections. However, these tests are generally restricted by antibody detection limits and cross reactivity (Schein, 1988; Bruning, 1996). Besides the CFT and IFAT, the enzyme-linked immunosorbent assay (ELISA) has also been used for the detection of *Theileria* infection. Various researchers have suggested this serological test as an alternative for increased specificity and sensitive detection of acute and latent infections, especially with the use of recombinant antigens (Tanaka et al., 1999; Xuan et al., 2001ab; Hirata et al., 2003).

Although regulatory control relies on serological tests to identify infected animals whose movement should be restricted, several other techniques including *in vitro* cultures, DNA probes, and polymerase chain reaction (PCR) have been used for the diagnosis of *T. equi*. Specific and sensitive molecular tools have helped the diagnosis of tick-borne diseases and contributed towards the elucidation of their epidemiology. A PCR test based on the ribossamal 16S RNA sequence has been used for the diagnosis of *T. equi* infection in horses showing 0.000083% parasitemia (Bashiruddin et al., 1999). Nested PCR has also been proven to be more sensitive in the detection of *T. equi* parasites than traditional microscope procedures, as it is able to detect the parasite in blood with an equivalent 0.000006% parasitemia (Nicolaiewsky et al., 2001). Additionally, it has been shown that *in vitro* techniques are capable of successfully detecting the carrier status of horses suspected of harboring *T. equi* (Holman et al., 1993; Zweygarth et al., 1997; Holman et al., 1998). Results from these studies suggests that *in vitro* culture could contribute to the identification of carrier animals and complement other methods of parasite detection, such as microscopy, serology, or PCR. The objective of this study was to investigate the effectiveness of PCR, nested PCR, and *in vitro* culture in the diagnosis of *T. equi* in horses exercised under controlled conditions and submitted to exercise-induced stress.

MATERIALS AND METHODS

Blood samples were collected from 15 apparently healthy Arabian horses from the Northeast region of the state of São Paulo, Brazil. Horses, previously conditioned to a high-speed equine treadmill, were exercised under controlled conditions. The animals were placed into two experimental groups: 30-day training schedule (G1) and 90-day training schedule (G2). Blood was taken while the animals were at rest and immediately prior to exercise-induced stress. Each horse was exercised at 4m/s for five minutes to warm up and then exercised for two minutes at 6m/s, 8m/s, and 10m/s on a six degree incline. Immediately after being exercised to exhaustion, additional blood sample was

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aseptically obtained from the jugular vein of each horse. The blood samples were collected in the presence of the EDTA anticoagulant and either used immediately for blood smears stained with Giemsa and *in vitro* culture or held at –20ºC for later use in PCR and nested PCR.

In vitro culture of parasites was carried out as previously described by Zweygarth et al. (1997). Blood samples were washed four times by centrifugation (700 x g, 10 minutes, at room temperature) and resuspended in a modified Vega y Martinez phosphate-buffered saline solution (Vega et al., 1985). The white blood cell layer overlaying the horse red blood cells (HRBC) was removed after each wash. After the final wash, the supernatant was removed and aliquots of 100µl of the cell pellet of each sample were distributed in a 24-well culture plates containing 900µl H-Y medium[1], supplemented with 20% normal adult horse serum, 100U penicillin per liter, 100µg streptomycin per milliliter, and 0.25µg amphotericin B[2] per milliliter. The cultures were incubated at 37ºC in a 2% oxygen, 5% carbon dioxide, and 93% nitrogen atmosphere. The medium was daily changed and 25µl of uninfected HRBC suspension was added to each well every three days. Giemsa-stained thin smears were performed every two days to monitor the appearance of *T. equi* in the cultures. Cultures were discontinued after 10 days and erythrocytes were stored in sterile microtubes at –20ºC until be used in molecular assays.

PCR assays were performed as previously described by Bashiruddin et al. (1999), with minor modifications. A DNA template was prepared from 200µl of each blood sample, which was washed three times by centrifugation with TE (10mM Tris-HCl pH 7.4, 1mM EDTA). The resulting pellets were resuspended in 200µl of water and incubated at 37ºC for 90 minutes with 0.1mg of proteinase K, 1% SDS, and 1% sarcosine. Then, DNA was extracted with phenol-chloroform-isoamilicum alcohol and precipitated in cold ethanol with 3M sodium acetate pH 5.2 at –70ºC for two hours. The final pellet was dissolved in 25µl of water and 3µl were used in a 50µl PCR mixture. All blood samples were also prepared using the Puregene

kit[3] according to the protocol of the manufacturer. The specific BEQF (5'-CATCGTTGCGGCTTGGTTGG-3') and BEQR (5'-CCAAGTCTCACACCCTATTT-3') primers, designed from the 16S rRNA sequence of *T. equi* (GenBank accession number Z15105), were used. PCR products were detected by electrophoresis on ethidium bromide stained 1% agarose gel.

Nested PCR was carried out according to Nicolaiewsky et al. (2001), in which nucleic acid was prepared as previously outlined The *EMAE-F* (5'-CCGCCCTTCACCTCGTTCTCAA-3')/*EMAE-R* (5'-TCTCGGCGGCATCCTTGACCTC-3') and *EMAI-F* (5'-CCGTCTCCGTTGACTTGGCCG-3')/*EMAI-R* (5'-GGACGCGCTTGCCTGGAGCCT-3') primers sets were chosen to flank the 396 and 102bp regions of the *EMA-1* gene sequence (GenBank accession number L13784), respectively.

The detection threshold test of *T. equi* DNA in blood samples with the specific 16S rRNA based PCR and *EMA-1* nested PCR was carried out according to Nicolaiewsky et al. (2001). Briefly, DNA (160ng) prepared from the blood of a *T. equi* experimentally infected horse (Jaboticabal, Brazil strain, GenBank accession number DQ250541) was serially diluted with blood from an uninfected horse. The initial parasitemia was 80% as measured in Giemsa stained thin blood smears. Serial dilutions were carried out in 10-fold steps to give final percentages of parasitized erythrocytes from 80 to 8.0×10^{-8}%.

Statistical analysis was performed using a chi-square test (χ^2 test), comparing positive rates obtained for *T. equi* by direct microscopic identification of the parasite in stained blood smears, *in vitro* culture, PCR, and nested PCR, before and after strenuous exercise. Agreement between the assays was assessed by *kappa* statistic. *Kappa* ranges from 1 (complete agreement) to 0 (agreement is equal to that expected by chance), whereas negative values indicate agreement less than that expected by chance. Benchmarks for interpreting *kappa* values were defined according to Everitt (1989): >0.81: almost perfect agreement; 0.61-0.80: substantial agreement; 0.41-0.61: moderate agreement; 0.21-0.40: fair agreement; 0-0.20: slight agreement; <0: poor agreement.

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RESULTS

Merozoites of *T. equi* were detected in blood smears of four horses under stress condition, from the 15 blood samples collected from G1 horses and examined before *in vitro* culture, while only two of these were also positive at rest. The results were similar in G2, except for a horse which was no longer positive by microscopic examination when at rest. Additionally, a horse negative at the first collection was deemed to be positive for *T. equi* after exercise (Tab. 1).

After culture, *T. equi* parasites were identified in 14 animals from G1. The time span required for culture diagnosis of *T. equi* varied, being most of the samples (n=12) positive within two days. A higher positive rate was observed in samples collected immediately after exercise, when compared to samples collected at rest. Parasites could be detected in all the samples taken during stress, which were positive by *in vitro* culture, whereas four of these samples were negative at rest. Similar results were obtained for samples collected from G2, in which 12 horses were positive for *T. equi* by culture diagnosis (Tab. 1). In only one of the samples which were positive before culture, no parasite could be detected until the end of the experiment. Similar to G1, the time span required for culture diagnosis of *T. equi* in G2 varied.

The experiment to determine the threshold of parasite DNA detection by PCR and nested PCR are shown in Fig. 1 and Fig. 2, respectively. The 16S rRNA based PCR detected *T. equi* DNA in blood with an equivalent $8 \times 10^{-5}\%$ parasitemia, while nested PCR based on the *EMA-1* gene detected $8 \times 10^{-6}\%$ parasitemia. Extra bands can be seen, but only at higher parasite DNA concentrations. No differences in the sensitivity of the PCR and nested PCR assays were observed when DNA extraction was performed either by phenol-chloroform or the Puregene kit. Additionally, no amplification was observed with DNA obtained from uninfected horse blood.

No amplified product was observed in any of the animals tested by 16S rRNA PCR. On the other hand, *T. equi* DNA was detected by nested PCR in all the horses, except for one which was negative at rest (Tab. 1). In relation to the samples collected after exercise, 13 horses were positive including the animal which was negative at rest. When DNA template was prepared from samples obtained after *in vitro* cultures, the nested PCR demonstrated that seven and five horses were negative for *T. equi* before and after exercise, respectively. No significant difference was observed in G1 and G2, considering the molecular detection of *T. equi* (Tab. 1).
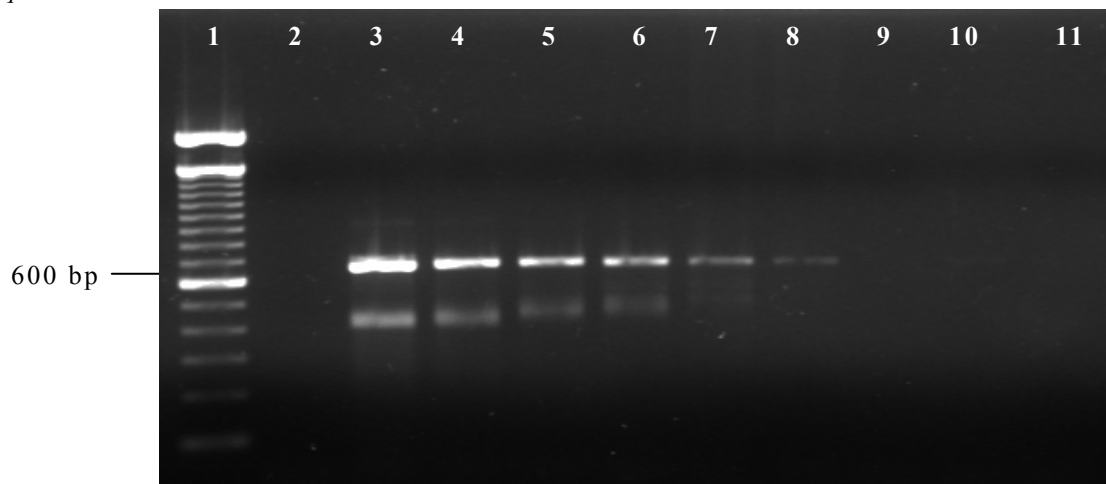

Figure 1. Detection threshold of *B. equi* DNA in infected equine blood using the 18S rRNA PCR. The PCR was performed on total DNA prepared from serially diluted blood of an infected horse (Jaboticabal strain, GenBank DQ250541) with initial parasitemia of 80%. Lane 1: 100 bp ladder DNA marker; lane 2: DNA from uninfected horse (negative control); lane 3: PCR with DNA from equine erythrocytes with parasitemia of 80%; lane 4: $8 \times 10^{-1}\%$; lane 5: $8 \times 10^{-2}\%$; lane 6: $8 \times 10^{-3}\%$; lane 7: $8 \times 10^{-4}\%$; lane 8: $8 \times 10^{-5}\%$; lane 9: $8 \times 10^{-6}\%$; lane 10: $8 \times 10^{-7}\%$; lane 11: $8 \times 10^{-8}\%$.
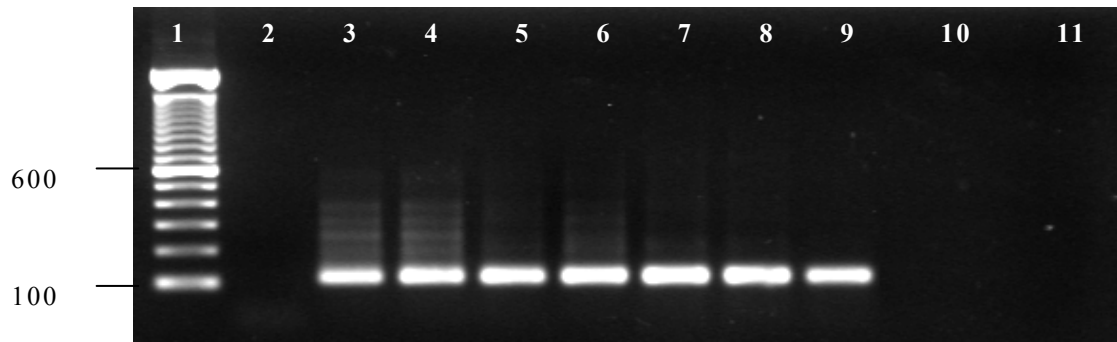
Figure 2. Detection threshold of *B. equi* DNA in infected equine blood using the *EMA-1* nested PCR. The PCR was performed on total DNA prepared from serially diluted blood of an infected horse (Jaboticabal strain, GenBank DQ250541) with initial parasitemia of 80%. Lane 1: 100 bp ladder DNA marker; lane 2: DNA from uninfected horse (negative control); lane 3: PCR with DNA from equine erythrocytes with parasitemia of 80%; lane 4: 8×10^{-1}%; lane 5: 8×10^{-2}%; lane 6: 8×10^{-3}%; lane 7: 8×10^{-4}%; lane 8: 8×10^{-5}%; lane 9: 8×10^{-6}%; lane 10: 8×10^{-7}%; lane 11: 8×10^{-8}%.

Table 1. Comparison of microscopic, *in vitro* culture, and molecular tests for the detection and diagnosis of *T. equi* in blood samples of naturally infected horses submitted to exercise after a 30-day (G1) and 90-day (G2) training schedules

Horse	Microscopy Before culture				In vitro culture																PCR (16S rRNA gene) Rest/Exercise		Nested PCR (EMA-1 gene)							
	Rest		Exercise		2th dc				4th dc				6th dc				10th dc						Before culture				After in vitro culture			
					Rest		Exercise		Rest		Exercise		Rest		Exercise		Rest		Exercise				Rest		Exercise		Rest		Exercise	
	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2	G1	G2
1	-	-	+	+	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-	-	-	+	+	+	+	-	-	+	+
2	-	-	-	-	+	+	+	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+
3	-	-	+	+	-	-	+	+	-	-	-	+	-	-	+	-	-	-	+	+	-	-	-	-	+	+	-	-	+	+
4	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
5	-	-	-	-	+	-	+	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	+	+	+	+	-	-	-	-
6	-	-	-	-	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	-	-	+	+
7	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-
9	-	-	-	-	+	-	+	-	+	-	+	+	+	-	+	+	-	-	-	+	-	-	+	+	+	+	-	-	-	-
10	-	-	-	-	+	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
11	-	-	+	+	-	-	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	+	+	+	+	+	+	-	-
12	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	-	-
13	-	-	-	+	-	+	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+
14	-	-	-	-	-	+	+	+	-	-	+	+	-	-	+	+	-	-	+	-	-	-	+	+	+	+	-	-	+	+
15	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	+	+	+
Positive Rate	2	1	4	5	7	5	11	7	5	5	8	9	3	0	8	7	2	0	2	2	0	0	14	14	13	13	8	8	10	10

dc = days of culture, (+) = positive animals, (-) = negative animals.
The following differences were significant (χ^2 test): positive nested PCR (before or after culture) reactions vs. positive microscopy (P<0.01), positive nested PCR (after culture) vs. positive microscopy (P<0.05), and positive microscopy vs. positive *in vitro* culture (P<0.01) for *T. equi* infections.

The detection and diagnosis of *T. equi* in blood samples of naturally infected horses submitted to exercise were compared. The differences were not significant among the samples collected at rest or after strenuous exercises in all the diagnostic methods performed in the present study. The following differences were significant (χ^2 test): positive nested PCR reactions (before or after culture) versus positive microscopy (P<0.01), positive nested PCR reactions (after culture) versus positive microscopy (P<0.05), and positive microscopy versus positive *in vitro* culture (P<0.01) for *T. equi* infections (Tab. 1).

Assessment of agreement among the assays by method of determining *kappa* statistic is shown in Tab. 2. The *kappa* coefficient ranged from –0.029 (microscopy and *in vitro* culture), indicating agreement less than is expected by chance, to 0.76 (culture and nested PCR before culture), suggesting fair agreement. After strenuous exercises (G1/G2), the *kappa* coefficient ranged from –0.09/–0.07 between microscopy and nested PCR (before culture), to 0.63/0.76 between culture and nested PCR (before culture).

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Table 2. Concordance among microscopic, *in vitro* culture, PCR, and nested PCR for the detection and diagnosis of *T. equi* in blood samples of naturally infected horses submitted to exercise after a 30-day (G1) and 90-day (G2) training schedules demonstrated by values of *kappa* statistic

Diagnosis Methods	*Kappa* coefficient			
	Rest		Exercise	
	G1	G2	G1	G2
Microscopy x nested PCR (before culture)	0.02	0.01	-0.09	-0.07
Microscopy x nested PCR (after culture)	-0.02	-0.13	0.31	0.40
Microscopy x *in vitro* culture	-0.29	-0.14	0.09	0.08
Microscopy x PCR	0.00	0.00	0.00	0.00
In vitro culture x PCR	0.00	0.00	0.00	0.00
In vitro culture x nested PCR (before culture)	0.25	0.4	0.63	0.76
In vitro culture x nested PCR (after culture)	0.18	0.05	-0.013	0.00
PCR x nested PCR (before culture)	0.00	0.00	0.00	0.00
PCR x nested PCR (after culture)	0.00	0.00	0.00	0.00
Nested PCR (before culture) x nested PCR (after culture)	0.15	0.15	0.12	0.12

Interpreting kappa values: >0.81: almost perfect agreement; 0.61-0.80: substancial agreement; 0.41-0.61: moderate agreement; 0.21-0.40: fair agreement; 0-0.20: slight agreement; <0: poor agreement.

DISCUSSION

The low positive rate for *T. equi* observed in the present study on the direct microscopic identification of the parasite by blood smears could be especially due to the low sensitivity of the method. Bose et al. (1995) reported the low sensitivity of this technique and suggest its use only in acute cases of the disease. Holman et al. (1998) suggested that negative results in stained blood smears could be due to time delay and handling between blood collection and slide preparation. Therefore, it should be mentioned therefore, that in the present study, the blood was immediately processed after the return from the treadmill, implicating in intervals of approximately three hours, a fact that would not justify the occurrence of false-negative results.

Theileria equi parasites were observed in cultured erythrocytes from 14 horses of G1 and 12 of G2. Such results demonstrated that the sensibility of this method is much higher than that related for blood smears and are in agreement with those recorded by Holman et al. (1998). Statistical analysis demonstrated significant differences (P<0.01) and *kappa* coefficient between culture and microscopy ranged from –0.29 to 0.09, showing poor agreement (Tab. 2). Although *T. equi* was observed in cultured erythrocytes from most of the sampled animals, the cultures did not become continuous and lower positive rates were observed toward the end of the experiment. It is

possible that the parasitemia was simply too low, or the parasites were not robust enough to allow *in vitro* establishment, or both. Holman et al. (1998) suggested that the successful cultivation of the parasite may be due to selection of a population that adapts to imposed conditions, and that some field sample parasites may be less adaptable to certain *in vitro* conditions than others. Considering that the samples of the present study were collected from Brazilian field horses and that very little information about the *T. equi* strains from Brazil are available, further studies should be carried out in order to elucidate such aspects. Unknown factors during field collection, handling, and shipping may also have affected the viability of the parasites (Holman et al., 1998). Zweygarth et al. (1997) demonstrated that although parasites are present in the blood, the sample volume to initiate culture may be too small to give rise to positive cultures.

Thus, the culture diagnostic technique used in the present study has been shown to be suitable for the diagnosis of *T. equi*, permitting the detection of several animals with positive results, especially carrier horses which frequently have extremely low circulating parasitemia. The differences observed in the time span for a positive diagnosis of *T. equi* could be related to the stage of the parasites or simply due to differential numbers of parasites in the samples (Holman et al., 1998). One of the disadvantages of this method is the incubation time required for identifying cultures as positive, which can range

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from two to 15 days. Additionally, the high cost of the reagents and the possibility of bacterial contamination makes this method unsuitable routine diagnosis or for epidemiological studies.

The occurrence of higher positive rates in stained blood smears and *in vitro* culture with samples collected at stress (Tab.1) could be explained by marked increases in erythrocyte numbers, packed cell volume, and mean corpuscular volume in horses submitted to training programmes and attributed to spleen contraction (Allen and Powell, 1983). The spleen of most mammals contracts during stress, resulting in a marked increase in blood erytrocytes, platelets and, to a lesser extent, blood leucocytes (Smith et al.,1989). Additionally, the effects of excitement or exercise seems to be most dramatic in horses, in which the packed cell volume can increase as much as 40% under stressful conditions (Kramer, 2000). Rose and Hodgson (1982) suggest that exercise programmes involving a large component of maximum exercise may stimulate increased erythrocyte production in order to transport oxygen more efficiently to the tissues.

The 16S PCR system was able to detect 8×10^{-5}% parasitaemia, equivalent to eight infected cells out of 10^7 erythrocytes. Bashiruddin et al. (1999) reported similar results in the molecular detection of *T. equi*, being able to detect an approximate 0.000083% parasitaemia in naturally infected horse blood. On the other hand, nested PCR using oligonucleotides designed on the sequence of a *T. equi* merozoite antigen gene (*EMA-1*) was able to detect the parasite in blood with an equivalent 0.000008% parasitaemia. Similar results were obtained by Nicolaiewsky et al. (2001) which reported detection limits of 0.000006% in a mock infection, equal to six infected cells out of 10^8 erythrocytes. The enhanced sensitivity of nested PCR observed in the present study when compared to the single-round 16S PCR could be explained by the fact that two separate rounds of amplification are required on a nested PCR and that the primers used codes for a highly intra-species conserved equi merozoite antigen (*EMA-1*) (Knowles et al., 1991; 1997). Rampersad et al. (2003) showed that nested PCR detected 3.6 times more *T. equi* infections than microscopic analysis and 2.2 times more than primary PCR.

All horses were negative for *T. equi* in 16S PCR tests, including animals which were positive in stained blood smears and *in vitro* culture (Tab.1). No positive results were also observed in the samples collected after *in vitro* culture. However, species-specific amplified products from positive control were visualized by agarose gel electrophoresis, demonstrating that both the nucleic acid preparation and the amplification conditions were correct for *T. equi*; therefore, validating the results of the present study. Canola et al. (2002) used this same PCR system for the detection of *T. equi* in naturally infected horses and also did not report positive results in any of the 18 horses evaluated. Although Bashiruddin et al. (1999) detected *T. equi* DNA in 22 horses using the 16S rRNA gene, animals had very high parasitaemia as confirmed by direct microscopic examination of stained blood smears.

Fourteen horses at rest and 13 animals after strenuous exercise tested positive with the nested PCR for *T. equi*. These results demonstrated that the nested PCR can easily detect *T. equi* DNA in blood from chronically infected horses, especially those which are negative by other direct diagnosis methods. It should be mentioned that all horses that tested negative by *in vitro* culture were positive by nested PCR, both at rest and stress, except horse number 15, which was positive only at rest.

The results obtained by nested PCR with samples collected after *in vitro* culture showed that only one of the horses with negative results turned out to be positive. Additionally, a lower number of samples were positive for *T. equi* when compared to the nested PCR carried out with samples collected before culture. It is possible that the sensitivity of the test may have been affected by several factors which inhibited DNA amplification. The samples submitted to nucleic acid preparation were obtained on the last day of *in vitro* culture, when low parasitaemias were observed, perhaps the number of parasites was not sufficient to be detected on the nested PCR. It also cannot be ruled out the possibility that high concentration of haemoglobin, which is known to interfere in PCR results (Loparev et al., 1991), may have affected the detection of parasites as the culture samples presented a high concentration of erythrocytes.

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No statistical differences were observed between nested PCR (before culture) and *in vitro* culture. The best agreement (*kappa* coefficient: 0.25/rest - 0.76/exercise) was observed between these diagnostic methods. These findings suggest that the nested PCR described herein is a useful tool for the detection of sub-clinical *T. equi* infections and that *in vitro* culture could be used as a supplement for other methods of parasite detection. Both methods should be considered for the diagnosis of equine piroplasmosis and could greatly aid in controlling the importation of infected animals, avoiding performance problems in racehorses. Additionally, the results of the present study have provided evidence that samples may be collected at rest or after strenuous exercises, not interfering in positive rates, although stress seems to have a positive effect on the detection of *T. equi* using blood smears and *in vitro* culture.

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