

RESEARCH NOTE

Detection of *Theileria equi* in spleen and blood of asymptomatic piroplasm carrier horses

Isabel B. Ribeiro¹, Antônio Carlos L. Câmara¹, Marta V. Bittencourt², Tatiana G. Marçola²,
Giane R. Paludo² and Benito Soto-Blanco^{1,3*}

¹Programa de Pós-Graduação em Ciência Animal, Universidade Federal Rural do Semi-árido (UFERSA), BR 110 Km 47, Mossoró, RN 59625-900, Brazil; ²Laboratório de Patologia Clínica Veterinária, Faculdade de Agronomia e Medicina Veterinária, Universidade de Brasília (UnB), Campus Universitário Darcy Ribeiro, ICC Sul, Caixa Postal 04508, Asa Norte, Brasília, DF 70910-970, Brazil; ³Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Caixa Postal 567, Belo Horizonte, MG 30123-970, Brazil

Abstract

This study aimed to determine whether asymptomatic horses naturally infected with *Theileria equi* retain infected erythrocytes in the spleen and whether the presence of the hemoparasite in this organ is associated with parasitemia. We collected samples from 25 adult horses without clinical signs of any disease. From each animal, we collected whole blood samples from the jugular vein and a splenic puncture blood sample. All samples were submitted to blood cell counts and detection of *Theileria* or *Babesia*. DNA extraction and PCR were performed in all samples for identification of piroplasm infection (*T. equi* and *B. caballi*). From the 25 horses evaluated for piroplasm detection by PCR, seven horses (28%) were positive in jugular vein blood but negative in splenic blood samples, five horses (20%) were positive in splenic blood samples but negative in jugular vein blood samples, and 13 horses (52%) were positive in both jugular vein and splenic blood samples. The hematological evaluation revealed anemia in 13 of 25 (52%) infected horses, lymphopenia in five (20%), neutrophilia in two (8%), neutropenia in one (4%), and thrombocytopenia in one (4%) infected horse. The present study demonstrated that several (20%) of the asymptomatic piroplasm carrier horses did not show parasitemia, but show infected erythrocytes in the spleen.

Keywords

Theileria equi, *Babesia caballi*, hemoparasites, piroplasm, carrier, parasitemia

Equine piroplasmosis is an intra-erythrocytic protozoan disease caused by *Theileria equi* and *Babesia caballi*, and it is transmitted by ticks of the Ixodidae family. This disease has high economic importance in the equine industry worldwide, causing damages ranging from a drop in performance to death (Bruning 1996; Friedhoff and Soule 1996). It is an endemic disease in the tropics, subtropics and warm temperate zones (De Waal 1992), with high prevalence in some countries. Moreover, efforts to control the spread of the disease affect the trade and transportation of horses (Bruning 1996; Friedhoff and Soule 1996), and piroplasm infection is the main impediment to the international transport of horses.

In acute and sub-acute piroplasmosis, horses may develop fever, anemia, icterus, hemoglobinuria and weakness; some cases may be fatal (De Waal *et al.* 1987). Despite the severity of acute infection, most animals can be clinically cured and then become asymptomatic carriers for years. The carriers may relapse in situations that lead to immunosuppression, such as stress (De Waal *et al.* 1987; Oladosu and Olufemi 1992; Rhalem *et al.* 2001). In these cases, the animals could show a poor performance, hyporexia and weight loss.

Although it is known that horses that are asymptomatic carriers can transmit the disease, little is known about the parasite's mechanism of persistence in the host, including where it resides within the host. It was determined that in some

*Corresponding author: benito.blanco@pq.cnpq.br

horses, *T. equi* and *B. caballi* can persist in bone marrow (Pitel *et al.* 2010). Furthermore, *T. equi* was detected in the liver, spleen, groin lymph node, lung, heart, bone marrow and brain of experimentally infected horses (Alhassan *et al.* 2007a). However, it has not been determined whether horses with piroplasm in tissues always present with parasitemia.

This study aimed to determine whether asymptomatic horses infected with *T. equi* retain infected erythrocytes in the spleen and whether the presence of the hemoparasite in this organ is associated with parasitemia.

The experimental design was carried out in strict accordance with the recommendations in the Ethical Principles for Use of Experimental Animals from Colégio Brasileiro de Experimentação Animal (COBEA), Brazil. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Universidade Federal Rural do Semi-Árido/UFERSA (Protocol Number: 2472). The study was conducted in the city of Mossoró, RN, Brazil, located at 5°11'S 37°20'W, 18 m altitude. The average annual temperature was 27°C, the mean annual relative humidity was around 70%, and the annual rainfall ranged from 450 to 650 mm. The prevailing weather in the region can be classified as tropical semi-arid, with the occurrence of two seasons throughout the year: the rainy season (usually from December to April) and the dry season.

Twenty-five (eighteen male and seven female) asymptomatic adult (2–16 years-old) client-owned mixed-bred horses were selected randomly. Samples were collected from each animal from September and October 2009. From each animal, we collected a whole blood sample from the jugular vein; another sample was obtained by splenic puncture. All samples were collected in tubes containing EDTA anticoagulant. Splenic punctures were performed using a 30 gauge x 8 cm needle. The spleen was accessed at the 17th intercostal space on the cranial edge of the left side after surgical clipping and aseptic preparation with application of povidone iodine to a 3 cm square region at the puncture site. After the antisepsis procedure, the needle was introduced at a 90° angle to the spleen. After introducing the needle, we collected approximately 0.2–0.5 mL of blood. Qualitative assessment for the detection of *Theileria* or *Babesia* was performed by optical microscopy (100x magnification) on Panoptic stained (InstantProv) blood smears.

DNA was extracted from all samples using commercial kits (Illustra™ GenomicPrep Blood Flow Mini Kit, GE Healthcare Life Sciences), following the manufacturer's instructions. DNA samples were kept at –20° C until PCR was performed. Water was used as negative control to determine whether there was contamination of any reagent, and as a positive control, we used the blood of an infected horse with parasitized erythrocytes in blood smears. Primer sequences used in this work are shown in Table I. All used sequences were identical to DNA sequences described for *B. caballi* and *T. equi* (Allsopp *et al.* 1994; see GenBank: Z15104 and Z15105). All reactions were performed in the same thermal cycler (MyCycler, Biorad ®).

Table I. Sequences of primers used for the identification of piroplasm in this study

Primer	Sequence
Bec-UF1	5'-GTTGATCCTGCCAGTAGTCA-3'
Bec-UR	5'-CGGTATCTGATCGTCTTCGA-3'
Bec-UF2	5'-TCGAAGACGATCAGATACCGTCG-3'
Cab-R	5'-CTCGTTCATGATTAGAATTGCT-3'
Equi-R	5'-TGCCTTAAACTTCCTTGCGAT-3'

Bec-UF1 and Bec-UF2 = universal forward primers; Bec-UR = universal reverse primer; Cab-R = *Babesia caballi*-specific reverse primer; Equi-R = *Theileria equi*-specific reverse primer (Alhassan *et al.* 2005).

To identify specific sequences of the 18S rRNA gene from both, *T. equi* and *B. caballi*, a common universal screening primer pair was used (Bec-UF1 and Bec-UR) as described by Alhassan *et al.* (2005). Additionally a multiplex PCR including Bec-UF2 as a universal forward primer and Cab-R and Equi-R as reverse primers specific for *B. caballi* and *T. equi*, respectively, was also tested in all samples (Alhassan *et al.* 2005). The reactions consisted of the following: 1X PCR buffer, approximately 10 ng DNA, 1.5 mM MgCl₂, 0.2 mM of each deoxynucleotide, 2.5 μM of each primer and 0.8 U Taq DNA polymerase in a final volume of 25 μL. The amplification conditions were an initial denaturation at 96°C for 10 min followed by 40 cycles of 96°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products from universal screening primers were electrophoresed on a 1.5% agarose gel, stained with ethidium bromide and visualized under UV light to confirm the presence of a product 867 or 913 bp in positive animals for *B. caballi* or *T. equi*, respectively. On the multiplex PCR the DNA fragments had 392 (*T. equi*) or 540 bp (*B. caballi*).

Results are presented as the mean with standard error. BioEstat version 5.0 software (MAMIRAUA, Tefê, Brazil) was used for all statistical analyses. The Mann-Whitney U test was used to test for significant differences in hematological data from horses with and without parasitemia. The Kruskal-Wallis test followed by a Student-Newman-Keuls multiple comparisons test was used to test for significant differences in hematological parameters within multiple groups (presence or absence of piroplasm in jugular vein and/or splenic blood samples by molecular detection). The level of statistical significance was set at P<0.05.

The qualitative assessment for detection of *Theileria equi* performed on blood smears revealed six (24%) horses positive on splenic blood smear and only three (12%) horses positive on jugular vein smear (Tables II and III). All positive horses in jugular vein smears were also positive in splenic smears. No *Babesia caballi* was found on any smear.

Results of the molecular detection of piroplasm are presented in Tables II and III. Seven horses (28%) were positive in jugular vein blood but negative in splenic blood

Table II. Results of piroplasm detection by qualitative assessment of smears and PCR analysis in jugular vein and splenic blood samples from asymptomatic horses

Splenic blood samples	Jugular blood smear	Splenic blood smear	Jugular blood PCR	Splenic blood PCR
Positives	3/25 (12%)	6/25 (24%)	20/25(80%)	18/25(72%)
Negatives	22/25 (88%)	19/25 (76%)	5/25(20%)	7/25 (28%)

samples, five horses (20%) were positive in splenic blood samples but negative in jugular vein blood samples, and 13 horses (52%) were positive in both jugular vein and splenic blood samples. All the positive results for piroplasm detection by optical microscopy were also positive by screening PCR method. *T. equi* was identified by PCR in eleven (44%) jugular vein blood samples and six (24%) splenic blood samples. Two animals that were negative using the species-specific primer (horses 1 and 20 in Table II) were found presenting *T. equi*-infected erythrocytes on smears evaluated by optical microscopy.

Babesia and *Theileria* organisms may be carried by clinically healthy horses for several years (Holbrook 1969, Schein 1988). The exact mechanism of their long persistence in the host is not fully understood. During the clinical phase of the

disease, the parasites may reach several tissues of the host. In fact, in experimentally infected horses, *T. equi* DNA was detected in the liver, spleen, groin lymph node, lungs, heart, bone marrow, and brain (Alhassan *et al.* 2007a). Recent studies have been shown that bone marrow may be a potential reservoir site of *T. equi* and *B. caballi* in clinically healthy horses (Alhassan *et al.* 2007a; Pitel *et al.* 2010). In our study, piroplasms were found in the spleen of asymptomatic horses, and this may be an important tissue supporting the persistence of the parasite in the organism. It remains to be determined whether the parasites replicate in the spleen to ensure their long survival in the host, or whether they enter a quiescent state. The possibility of erythrocytic multiplication of piroplasm in the spleen should be considered and investigated in future studies.

Table III. Results of PCR analysis using *Babesia/Theileria* (BT) primers and species-specific primers in jugular vein and splenic blood samples from asymptomatic horses

Horse	Jugular blood samples			Splenic blood samples		
	Qualitative	BT primers	Species-specific	Qualitative	BT primers	Species-specific
01	+	+	-	+	-	-
02	-	+	<i>T. equi</i>	+	+	-
03	-	+	-	-	-	-
04	-	+	<i>T. equi</i>	-	+	-
05	-	+	<i>T. equi</i>	-	+	<i>T. equi</i>
06	-	-	-	-	+	-
07	-	+	<i>T. equi</i>	-	-	-
08	-	+	-	-	+	-
09	+	+	<i>T. equi</i>	+	+	-
10	-	+	-	-	+	-
11	-	+	-	-	+	-
12	-	-	-	-	+	-
13	+	+	<i>T. equi</i>	+	+	<i>T. equi</i>
14	-	+	-	-	+	-
15	-	-	-	-	+	-
16	-	+	<i>T. equi</i>	-	-	-
17	-	-	-	-	+	-
18	-	+	<i>T. equi</i>	-	-	-
19	-	+	-	-	+	<i>T. equi</i>
20	-	-	-	+	+	-
21	-	+	<i>T. equi</i>	-	+	<i>T. equi</i>
22	-	+	-	-	-	-
23	-	+	-	+	+	<i>T. equi</i>
24	-	+	<i>T. equi</i>	-	+	<i>T. equi</i>
25	-	+	<i>T. equi</i>	-	-	-

The hemoparasite may be present in the spleens of asymptomatic horses without parasitemia assigned as chronic carriers as well as those in the acute phase of the disease and also been detectable by jugular blood smears. This finding is particularly beneficial for diagnostic identification of carrier horses from endemic regions because the piroplasm could be undetected in the blood of carrier animals that could infect ticks and transmit the disease to other horses (Holman *et al.* 1997). The transport of these carrier horses may result in the dissemination of the parasites in *T. equi*-free countries. Our results reported that this PCR method revealed to fail in detecting the *T. equi* DNA in two horses (Horses 1 and 20) confirmed to be infected using the blood smear method. Failure to detect the etiologic agents by PCR is most probably due to the parasites clearance from the circulating blood by the host. Horses are known sometimes to adapt to infection and may appear only in certain circumstances, such as under stress or severe immune suppression resulting in relapse of the infection (Abutarbush *et al.* 2012). In addition, parasites DNA in our study blood samples may have been present in levels below the sensitivity threshold of the conventional PCR which was used.

Our study showed the presence of *T. equi* parasitized erythrocytes in the spleen of horses. However, because we just tested the spleen it is possible that the parasite might be present in other organs like the results of Alhassan *et al.* (2007a) in experimentally infected horses. However, *Babesia canis* and *Babesia bovis* parasitized erythrocytes are able to escape the passage through the spleen (Allsopp *et al.* 1994; Mehlhorn and Schein 1998; Schettters *et al.* 1998; Botteon *et al.* 2005; Carcy *et al.* 2006). Seven of the evaluated horses were found to present the parasite in whole blood, but not in the splenic blood. Thus, it is possible that the escape of parasitized cells from the spleen also occurs in horses, but this hypothesis remains to be tested.

Results showed that PCR is a more sensitive and accurate diagnostic method than microscopic examination of blood smears. The microscopic examination of blood smears is useful for the diagnosis of acute piroplasmosis in horses, but its efficiency is limited in cases of low parasitemia (Böse *et al.* 1995; Seifi *et al.* 2000; Abutarbush *et al.* 2012). In cases of low parasitemia, hemoparasite DNA detection by PCR is extremely efficient for diagnosis because this technique has high specificity and sensitivity (Bashiruddin *et al.* 1999; Ramperasad *et al.* 2003; Alhassan *et al.* 2007b).

Theileria equi was identified in most cases (56%), but the species was not identified in the other cases. This may be attributed to the limit of detection of the used PCR method, which was estimated as 0.18 parasites for *B. caballi* and 0.018 parasites for *B. equi*, but at mixed infection it was 1.8 parasites for *B. caballi* and 0.18 parasites for *B. equi* (Alhassan *et al.* 2005). However, the most probable is the occurrence of sequence variations in the 18S rRNA gene in the parasites, as it was observed in *T. equi* from several countries including Spain (Criado-Fornelio *et al.* 2004), South Africa (Bhoora *et al.*

2009), Sudan (Salim *et al.* 2010) and Greece (Kouam *et al.* 2010). Furthermore, in our study two parasitized horses that were negative using the species-specific primer were found presenting *T. equi*-infected erythrocytes on smears evaluated by optical microscopy. Another possibility is that some horses were infected with other piroplasms species. In fact, it has been observed that horses can carry *B. bovis* and *B. bigemina* DNA (Bulling *et al.* 2007), which are highly prevalent parasites in cattle at Northeastern region of Brazil (Costa *et al.* 2011).

The present study demonstrated that some of the asymptomatic *T. equi* carrier horses did not show parasitemia but presented sequestered infected erythrocytes in the spleen.

References

- Abutarbush S.M., Alqawasmeh D.M., Mukbel R.M., Al-Majali A.M. 2012. Equine babesiosis: seroprevalence, risk factors and comparison of different diagnostic methods in Jordan. *Transboundary and Emerging Diseases*, 59, 72–78. DOI: 10.1111/j.1865-1682.2011.01244.x.
- Alhassan A., Govind Y., Tam N.T., Thekisoe O.M.M., Yokoyama N., Inoue N., Igarashi I. 2007a. Comparative evaluation of the sensitivity of LAMP, PCR and *in vitro* culture methods for the diagnosis of equine piroplasmosis. *Parasitology Research*, 100, 1165–1168. DOI: DOI: 10.1007/s00436-006-0430-6.
- Alhassan A., Iseki H., Kim C., Yokoyama N., Igarashi I. 2007b. Comparison of polymerase chain reaction methods for the detection of *Theileria equi* infection using whole blood compared with pre-extracted DNA samples as PCR templates. *Tropical Animal Health and Production*, 39, 369–374. DOI: 10.1007/s11250-007-9025-1.
- Alhassan A., Pumidonming W., Okamura M., Hirata H., Battsetseg B., Fujisaki K., Yokoyama N., Igarashi I. 2005. Development of a single-round and multiplex PCR method for the simultaneous detection of *Babesia caballi* and *Babesia equi* in horse blood. *Veterinary Parasitology*, 129, 43–49. DOI: 10.1016/j.vetpar.2004.12.018.
- Allsopp M.T.E.P., Cavalier-Smith T., De Waal D.T., Allsopp B.A. 1994. Phylogeny and evolution of the piroplasms. *Parasitology*, 108, 147–152. DOI: 10.1017/S0031182000068232.
- Bashiruddin J., Camma C., Rebelo E. 1999. Molecular detection of *Babesia equi* and *Babesia caballi* in horse blood by PCR amplification of part of the 16S rRNA gene. *Veterinary Parasitology*, 84, 75–83. DOI: 10.1016/S0304-4017(99)00049-7.
- Bhoora R., Franssen L., Oosthuizen M.C., Guthrie A.J., Zweggarth E., Penzhorn B.L., Jongejan F., Collins N.E. 2009. Sequence heterogeneity in the 18S rRNA gene within *Theileria equi* and *Babesia caballi* from horses in South Africa. *Veterinary Parasitology*, 159, 112–120. DOI: 10.1016/j.vetpar.2008.10.004.
- Böse R., Jorgensen W.K., Dalgliesh R.J., Friedhoff K.T., deVos A.J. 1995. Current state and future trends in the diagnosis of babesiosis. *Veterinary Parasitology*, 57, 61–74. DOI: 10.1016/0304-4017(94)03111-9.
- Botteon P.T.L., Botteon R.C.C.M., Reis T.P., Massard C.L. 2005. Babesiose em cavalos atletas portadores. *Ciência Animal*, 35, 1136–1140.
- Bruning E. 1996. Equine piroplasmosis: An update on diagnosis, treatment, and prevention. *British Veterinary Journal*, 152, 139–151. DOI: 10.1016/S0007-1935(96)80070-4.
- Bulling A., Criado-Fornelio A., Asenzo G., Benitez D., Barba-Carretero J.C., Florin-Christensen M. 2007. A quantitative PCR assay for the detection and quantification of *Babesia bovis*

- and *B. bigemina*. *Veterinary Parasitology*, 147, 16–25. DOI: 10.1016/j.vetpar.2007.03.031.
- Carcy B., Précigout E., Schetters T., Gorenflot A. 2006. Genetic basis for GPI-anchor merozoite surface antigen polymorphism of *Babesia* and resulting antigenic diversity. *Veterinary Parasitology*, 138, 33–49. DOI: 10.1016/j.vetpar.2006.01.038.
- Costa V.M.M., Rodrigues A.L., Medeiros J.M.A., Labruna M.B., Simões S.V.D., Riet-Correa F. 2011. Tristeza parasitária bovina no Sertão da Paraíba. *Pesquisa Veterinária Brasileira*, 31, 239–243. DOI: 10.1590/S0100-736X2011000300009.
- Criado-Fornelio A., González-del-Río M.A., Buling-Saraña A., Barba-Carretero J.C. 2004. The “expanding universe” of piroplasms. *Veterinary Parasitology*, 119, 337–345. DOI: 10.1016/j.vetpar.2003.11.015.
- De Waal D.T. 1992. Equine piroplasmosis: a review. *British Veterinary Journal*, 148, 6–14. DOI: 10.1016/0007-1935(92)90061-5.
- De Waal D.T., Van Heerden J., Potgieter F.T. 1987. An investigation into the clinical pathological changes and serological response in horses experimentally infected with *Babesia equi* and *Babesia caballi*. *Onderstepoort Journal of Veterinary Research*, 54, 561–568.
- Friedhoff K.T., Soule C. 1996. An account on equine babesioses. *OIE Revue Scientifique et Technique*, 15, 1191–1201.
- Holbrook A.A. 1969. Biology of equine piroplasmosis. *Journal of the American Veterinary Medical Association*, 155, 453–454.
- Holman P.J., Hietala S.K., Kayashima L.R., Olson D., Waghela S.D., Wagner G.G. 1997. Case report: Field-acquired subclinical *Babesia equi* infection confirmed by *in vitro* culture. *Journal of Clinical Microbiology*, 35, 474–476.
- Kouam M.K., Kantzoura V., Masuoka P.M., Gajadhar A.A., Theodoropoulos G. 2010. Genetic diversity of equine piroplasms in Greece with a note on speciation within *Theileria* genotypes (*T. equi* and *T. equi* like). *Infection, Genetics and Evolution*, 10, 963–968. DOI: 10.1016/j.meegid.2010.06.008.
- Mehlhorn H., Schein E. 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. *Parasitology Research*, 84, 467–475. DOI: 10.1007/s004360050431.
- Oladosu L.A., Olufemi B.E. 1992. Haematology of experimental babesiosis and ehrlichiosis in steroid immunosuppressed horses. *Journal of Veterinary Medicine Series B*, 39, 345–352. DOI: 10.1111/j.1439-0450.1992.tb01179.x.
- Pitel P.H., Scriver T., Léon A. 2010. Molecular detection of *Theileria equi* and *Babesia caballi* in the bone marrow of asymptomatic horses. *Veterinary Parasitology*, 170, 182–184. DOI: 10.1016/j.vetpar.2010.01.043.
- Rampersad J., Cesa E., Campbell M.D., Samlal M., Ammons D. 2003. A field evaluation of PCR for the routine detection of *Babesia equi* in horses. *Veterinary Parasitology*, 114, 81–87. DOI: 10.1016/S0304-4017(03)00129-8.
- Rhalem A., Sahibi H., Lasri S., Johnson W.C., Kappmeyer L.S., Hamidouch A., Knowles D.P., Goff W.L. 2001. Validation of a competitive enzyme-linked immunosorbent assay for diagnosing *Babesia equi* infections of Moroccan origin and its use in determining the seroprevalence of *B. equi* in Morocco. *Journal of Veterinary Diagnostic Investigation*, 13, 249–251. DOI: 10.1177/104063870101300311.
- Salim B., Bakheit M.A., Kamau J., Nakamura I., Sugimoto C. 2010. Nucleotide sequence heterogeneity in the small subunit ribosomal RNA gene within *Theileria equi* from horses in Sudan. *Parasitology Research*, 106, 493–498. DOI: 10.1007/s00436-009-1691-7.
- Schein E. 1988. Equine babesiosis. In: (Ed. M. Ristic) *Babesiosis of Domestic Animals and Man*. CRC Press, Boca Raton, 197–208.
- Schetters T.P.M., Kleuskens J., Scholtes N., Gorenflot A. 1998. Parasite localization and dissemination in the *Babesia*-infected host. *Annals of Tropical Medicine and Parasitology*, 92, 513–519. DOI: 10.1080/00034989859483.
- Seifi H.A., Mohri M., Sardari K.A. 2000. Mixed infection of *Babesia equi* and *Babesia caballi* in a racing colt: A report from Iran. *Journal of Equine Veterinary Science*, 20, 858–860. DOI: 10.1016/S0737-0806(00)80117-3.