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Comparison of direct blood smear methods to detect piroplasms in wandering horses from Midwest Brazil[1]

Arthur M.S.V. Pinto[2], Verônica L.S. Argenta[2], Patrícia C. Duarte[3], Tayná C.M. Fino[2], Benito Soto-Blanco[3] and Antonio Carlos L. Câmara[2]*

ABSTRACT.- Pinto A.M.S.V., Argenta V.L.S., Duarte P.C., Fino T.C.M., Soto-Blanco B. & Câmara A.C.L. 2024. **Comparison of direct blood smear methods to detect piroplasms in wandering horses from Midwest Brazil**. *Pesquisa Veterinária Brasileira 44:e07496, 2024*. Hospital Escola de Grandes Animais, Universidade de Brasília, Área Especial SRB, Galpão 4, Granja do Torto, DF 70636-200, Brazil. E-mail: aclcamara82@gmail.com

Equine piroplasmosis is an apicomplexan hemoprotozoan-caused disease that affects equids worldwide. Horses that survive piroplasmosis can become asymptomatic carriers for the rest of their lives. The present study aimed to determine the frequency of piroplasms and to compare three different blood smear tests (jugular, peripheral, and splenic blood) to detect piroplasms in asymptomatic wandering horses seized by the "Secretaria de Estado da Agricultura, Abastecimento e Desenvolvimento Rural do Distrito Federal" (SEAGRI-DF), Midwest region of Brazil. Of the 100 horses evaluated, 38 were diagnosed positive for piroplasm (38%), with the etiological agents found at the jugular blood smear in 11% (11/100) of horses, peripheral blood smear in 13% (13/100), and splenic blood smear in 38% (38/100). Piroplasm-positive horses showed anemia, neutrophilia, and lymphopenia, but the hematological changes did not differ statistically between positive and negative horses. In summary, the stray horses evaluated showed a high incidence of piroplasm (38%). All positive horses presented one of the etiologic agents in the splenic blood, but some did not show the parasite in the jugular blood smear (27/38, 71%) or the peripheral blood smear (25/38, 65.8%). Thus, the splenic blood was shown to be the best sample to determine the presence of piroplasm in wandering horses. As it is a low-cost and easy-to-perform test, it can be included in the routine diagnosis of equine piroplasmosis, helping to monitor the prevalence of piroplasms in places where molecular techniques are not accessible.

INDEX TERMS: Piroplasmosis, piroplasm, equine, blood smear, splenic blood, splenic puncture.

RESUMO.- [**Comparação de métodos de esfregaço sanguíneo direto para detecção de piroplasmídeos em equinos errantes do Centro-Oeste do Brasil.**] A piroplasmose é uma doença causada por hemoprotozoários apicomplexos que afeta equídeos em todo o mundo. Equinos que sobrevivem à piroplasmose podem se tornar portadores assintomáticos pelo resto de suas vidas. O presente estudo teve como objetivo determinar a frequência de piroplasmas em equinos errantes apreendidos pela Secretaria de Estado da Agricultura, Abastecimento e Desenvolvimento Rural do Distrito Federal (SEAGRI-DF), região Centro-Oeste do Brasil, e comparar três diferentes exames diagnósticos de esfregaços sanguíneos: sangue jugular, periférico e esplênico. Dos 100 equinos avaliados, 38 foram diagnosticados positivos para piroplasmídeos, sendo o agente etiológico encontrado no esfregaço de sangue jugular em 11% (11/100), no esfregaço de sangue periférico em 13% (13/100) e no esfregaço de sangue esplênico em 38% (38/100) dos equinos. Os cavalos positivos apresentaram anemia, neutrofilia e linfopenia, mas as alterações hematológicas não apresentaram diferença estatística entre cavalos positivos e negativos. Em resumo, os equinos errantes avaliados apresentaram alta incidência de piroplasmídeos (38%). Todos os cavalos positivos apresentaram um dos agentes etiológicos no esfregaço sangue esplênico, mas alguns não apresentaram o parasita no esfregaço de sangue jugular (27/38, 71%) ou no esfregaço de sangue periférico (25/38, 65.8%). Assim, o sangue esplênico mostrou-se a melhor amostra

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[2]Hospital Escola de Grandes Animais, Faculdade de Agronomia e Medicina Veterinária (FAV), Universidade de Brasília (UNB), Área Especial SRB, Galpão 4, Granja do Torto, DF 70636-200, Brazil. *Corresponding author: aclcamara82@gmail.com

[3]Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais (UFMG), Av. Pres. Antônio Carlos 6627, Belo Horizonte, MG 30123-970, Brazil.

para determinar a presença de piroplasmídeos em equinos errantes. Por ser um exame de baixo custo e fácil execução, pode ser incluído na rotina diagnóstica da piroplasmose equina, auxiliando no monitoramento da doença em locais onde as técnicas moleculares não são acessíveis.

TERMOS DE INDEXAÇÃO: Piroplasmose, piroplasma, equinos, esfregaço sanguíneo, sangue esplênico, punção esplênica.

INTRODUCTION

Equine piroplasmosis is an apicomplexan hemoprotozoan-caused disease that affects horses, donkeys, mules and zebras. The causative agents are the intraerythrocytic protozoa *Babesia caballi* and *Theileria equi* (formerly called *Babesia equi*). Transmission of protozoa occurs through certain species of ticks. However, these pathogens can also be mechanically transmitted by the use of improperly disinfected needles, syringes, and surgical instruments or through blood transfusion from an infected donor horse (Pelzel-McCluskey & Traub-Dargatz 2015).

The disease is endemic in tropical and temperate regions with prevalent tick vectors. Due to piroplasmosis, the international commercialization and transit of horses are restricted in some countries. Despite the presence of the vector, several countries do not present the disease in an endemic form and control the entry of possible asymptomatic infected animals (Onyiche et al. 2019).

Peracute and acute clinical signs may include fever, jaundice, anemia, hemoglobinuria, inflammatory myopathy (Pasolini et al. 2018), and occasionally death (Onyiche et al. 2019). Horses with chronic piroplasmosis may present inappetence, weight loss, and poor performance (Pelzel-McCluskey & Traub-Dargatz 2015). Vertical transmission of *T. equi* has been documented and reported to result in abortion (Sousa et al. 2017) and neonatal infection (Onyiche et al. 2019). Horses that survive piroplasmosis can become asymptomatic piroplasm carriers, carrying *B. caballi* for years and *T. equi* for the rest of their lives (Onyiche et al. 2019). It is important to recognize that many chronically infected horses may show no clinical abnormalities, appearing clinically and hematologically normal. These animals are termed carriers and are reservoirs for tick-borne and iatrogenic transmission (Pelzel-McCluskey & Traub-Dargatz 2015).

The present study aimed to determine the frequency of piroplasms and to compare direct smear methods (jugular, peripheral and splenic blood) as a screening trial test for the detection of piroplasms in wandering asymptomatic horses from the roads of the Distrito Federal, Midwest region of Brazil.

MATERIALS AND METHODS

Ethical approval. The equids detailed in this study were wandering horses seized by the "Secretaria de Estado da Agricultura, Abastecimento e Desenvolvimento Rural do Distrito Federal" (SEAGRI-DF). The management of the cases was not altered by the study, and no ethical approval was obtained. The authors confirm that the study has followed the 1964 Declaration of Helsinki guidelines and its later amendments.

Data from routine analysis files were evaluated on 100 crossbred horses (59 males and 41 females). The horses came from operations to seize wandering animals from the roads of the Distrito Federal, Midwest region of Brazil, from September 2018 to January 2020. The age of the horses was determined by evaluating the dental arch, and the ages varied from one to 20 years.

Blood samples were obtained from the jugular vein, peripheral blood, and spleen of each animal. The horses were restrained for collections using halters, restraint trunks or derivative restraints if necessary. However, no sedative or local anesthetic drugs were used on any animal. Routine trichotomy and antisepsis were performed before each puncture.

Whole blood samples were obtained through jugular venipuncture using 25 x 7mm needles coupled to 10mL syringes and then transferred to tubes with ethylenediaminetetraacetic acid (EDTA) to perform the hematological profile and prepare the blood smear. For peripheral blood smears, the tip of the ear was shaved and pierced with a 25 x 7mm needle, avoiding vascularized areas, and after compression, the drop of blood obtained was used to prepare the smear. For splenic puncture, 30 x 8mm needles were used coupled to 10mL heparinized syringes (0.25mL of heparin solution), with this puncture being performed at a 90° angle on the 16th (for foals) or 17th (for adult horses) left intercostal space at the level of the iliac tuberosity. An ultrasound examination was necessary for seven horses to locate the spleen and properly collect the splenic blood sample.

Blood smears were prepared immediately after collection, then stained with rapid panoptic, and the slides were washed in running water (Meyer et al. 1995). The smear slides were examined under an optical microscope with 100x magnification throughout, observing the presence of piroplasmid. For each animal, three slides were prepared and evaluated. Blood count determination was performed using a hematological analyzer (pocH-100Iv-Diff, Sysmex, Kobe, Japan). Simultaneously, total plasma proteins were measured by refractometry on a portable refractometer, and the fibrinogen concentration was determined by the heat precipitation method performed on anticoagulated samples according to Millar's technique (Millar et al. 1971).

The hematological analysis values of positive and negative horses for babesiosis were statistically compared using the BioEstat v.5.0 software. The three smear methods were compared using Pearson's Chi-square test for positive and negative results frequencies. Data normality was assessed using the Shapiro-Wilk test for the hematological values, and a comparison between means was performed using Student's t-test. The frequencies of hematological changes in positive and negative horses were compared using the Fisher's Exact test. The significance level was set at $p<0.05$.

RESULTS

Of the 100 horses evaluated, 38 were diagnosed positive for piroplasm (*Babesia caballi* or *Theileria equi*), with the etiological agent found in at least one type of blood smear. The percentage of positives in each technique was 11% (11/100) in jugular blood smear, 13% (13/100) in peripheral blood smear, and 38% (38/100) in splenic blood smear. All positive horses presented intraerythrocytic piroplasms. The frequencies of positive results were significantly different when comparing the splenic blood method with the other two methods ($p<0.05$, Pearson's chi-square test). Still, there was no significant difference between the jugular and peripheral blood smear methods ($p>0.05$, Pearson's chi-square test). In all 38 positive horses, the parasite was found in the splenic blood smear, with 19 horses having the parasite only in the splenic sample. Only five horses tested positive in all samples. In contrast, six tested positive only in the jugular and splenic

blood smears, and eight only in the peripheral and splenic blood smears (Fig.1). The characterization of the piroplasm was not performed in this study since the main goal was to verify the feasibility of blood smear (especially splenic blood) as a screening diagnostic tool for infected or carrier's horses.

The results of the hematological evaluations are presented in Table 1. No statistically significant difference was found between the values obtained in positive and negative horses (p>0.05, Student's t-test). The individual values of each parameter in each horse were compared with the reference values for the species (Table 2). However, no statistically significant difference was found between the frequencies of hematological changes in positive and negative horses (p>0.05, Fisher's Exact test).

DISCUSSION

Parasitemia levels can be very low in horses that have recovered from clinical disease and even during the acute phase of infection. Thus, examination of blood smears may be impaired, especially in chronic carriers (Pelzel-McCluskey & Traub-Dargatz 2015). In this way, many routine analyses performed may give false-negative results. A possible example was a competition horse taken to the Tokyo 2020 Olympic Games, probably infected before arriving in Japan. Even with the control for international transport, some horses may present negative results in pre-boarding assessments (Aida et al. 2023).

As piroplasm may be restricted to the spleen, both jugular and peripheral blood analyses from asymptomatic carrier horses may present negative results (Ribeiro et al. 2013). This study was performed on asymptomatic wandering horses that were probably chronic carriers of piroplasm. Nevertheless, 38% of the evaluated wandering horses were positive for piroplasm in the splenic blood smear. Examination of jugular and peripheral blood smears revealed the presence of piroplasm in 29% (11/38) and 34.2% (13/38), respectively, of horses that had the parasite in the spleen. Therefore, the effectiveness of diagnosing equine piroplasmosis by direct methods such as blood smears increases when using spleen blood as a sample.

Several types of serologic (complement fixation test, indirect immunofluorescent antibody test, competitive inhibition enzyme-linked immunosorbent assay – ELISA) and molecular tests (polymerase chain reaction – PCR) are available for the detection of the antibodies against *Babesia caballi* and *Theileria equi* or the parasite itself (Pelzel-McCluskey & Traub-Dargatz 2015, Vieira et al. 2018). The use of such techniques helps overcome the challenges faced in the detection of low parasitemia of equine piroplasm parasite infections, especially in endemic areas (Coluccia et al. 2024, Mohammad-Naseri et al. 2024, Raza et al. 2024). However, the cost of serologic or molecular testing might make the study unfeasible when we deal

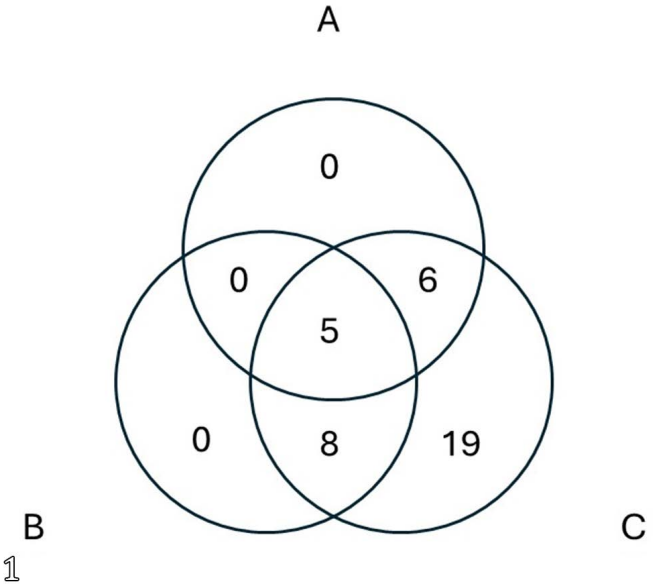

Fig.1. Venn diagram showing the positive results for piroplasm using jugular (A), peripheral (B), and splenic (C) blood smear methods in wandering horses from the Midwest region of Brazil.

Table 1. Hematological values of positive and negative horses for piroplasms

Parameter	Positives (n=38)	Negatives (n=62)	Reference range*
PCV (%)	30.1±6.53	31.0±5.42	24 – 53
RBC (x 10^6/µl)	6.92±1.51	6.64±1.18	5.5 – 12.9
Hemoglobin (g/dl)	11.8±2.3	12.2±1.75	8.0 – 19.0
WBC (/µl)	9,089.5±2,375.8	8,680.7±2,379.3	5,000 – 14,300
Neutrophils (/µl)	5,506.1±2,368.9	5,533.2±2,092.7	2,260 – 8,580
Lymphocytes (/µl)	2,493.8±1,096.4	2,484.7±1,281.8	1,500 – 7,700
Monocytes (/µl)	318.5±170.4	323.6±212.3	0 – 1000
Eosinophils (/µl)	274.4±273.5	319.1±282.8	0 – 1000
Basophils (/µl)	52.7±70.0	60.6±66.1	0 – 290
Platelets (x 10^3/µl)	172.5±38.8	179.1±44.3	100 – 350
Plasma proteins (g/dl)	7.93±0.86	7.91±0.70	5.80 – 8.70
Fibrinogen (mg/dl)	459.5±155.4	391.9±182.4	100 – 400

* Kramer (2006); PCV = packed cell volume, RBC = red blood cells, WBC = white blood cells; Data showed no significant difference between groups (p>0.05, Student´s t-test).

Table 2. Frequency of hematological changes observed in positive and negative horses for piroplasms

Parameter		Positives (n=38)	Negatives (n=62)
PCV	Reduced	6 (15.8%)	6 (9.7%)
RBC (x 10^6/µl)	Reduced	5 (13.2%)	11 (17.7%)
Hemoglobin (g/dl)	Reduced	3 (7.9%)	0
WBC (/µl)	Reduced	0	1 (1.6%)
	Increased	1 (2.6%)	1 (1.6%)
Neutrophils (/µl)	Increased	6 (15.8%)	5 (8.1%)
Lymphocytes (/µl)	Reduced	6 (15.8%)	15 (24.2%)
Eosinophils (/µl)	Increased	1 (2.6%)	2 (3.2%)
Platelets (x 10^3/µl)	Reduced	0	1 (1.6%)
Plasma proteins (g/dl)	Reduced	1 (2.6%)	0
	Increased	5 (13.2%)	7 (11.3%)
Fibrinogen (mg/dl)	Increased	16 (42.1%)	19 (30.6%)

PCV = packed cell volume, RBC = red blood cell, WBC = white blood cell; Data showed no significant difference between groups (p>0.05, Fisher's Exact test).

with wandering horses. Therefore, the use of a splenic blood smear is a cheap and easy-to-perform trial test in such cases. Additionally, using splenic blood to perform molecular testing is also associated with a higher accuracy (Ribeiro et al. 2013).

As a tick-borne disease, equids residing in endemic regions have a higher potential for tick exposure, and the vector's lack of control (environmental and *in vivo*) enhances the chance of transmission to susceptible hosts. It is important to identify equine piroplasmids in different hosts, such as dogs and tapirs (Criado-Fornelio et al. 2004) since the coexistence between wandering equids and local fauna is an important epidemiological factor for the dissemination of equine piroplasmosis. In addition, wandering equids (horses, mules, and donkeys) are important hosts of different etiologic agents, such as *Trypanosoma vivax* (Rodrigues et al. 2015) and piroplasmids (Machado et al. 2012, Vieira et al. 2018). That makes it necessary to constantly monitor these horses to detect carrier animals and prevent the spread of equine piroplasmosis and other diseases, including those with zoonotic potential.

The most common hematological changes observed in cases of piroplasmosis in horses are anemia and thrombocytopenia (Zobba et al. 2008, Ribeiro et al. 2013, Mahmoud et al. 2016, Osman 2017). Other hematological changes include leukocytosis (Zobba et al. 2008) or leukopenia (Raza et al. 2024), neutrophilia or neutropenia, lymphopenia (Ribeiro et al. 2013, Mahmoud et al. 2016) or lymphocytosis (Raza et al. 2024). In the present study, anemia, neutrophilia, and lymphopenia were also found in positive horses. However, these changes cannot be associated with piroplasmosis because there was no statistically significant difference between positive and negative horses for any of the parameters evaluated. This finding may be associated with the unsatisfactory nutritional and sanitary conditions in which draft or wandering horses might be kept (Rezende 2016), especially the high prevalence of gastrointestinal parasites (Costa et al. 2018).

CONCLUSION

The wandering horses evaluated showed a high incidence of piroplasm (38%). All positive horses presented the piroplasm in the splenic blood, but some did not show the parasite in the jugular blood smear (27/38, 71%) or the peripheral blood smear (25/38, 65.8%). Thus, the splenic blood was the best sample to determine the presence of piroplasms in horses. As it is a low-cost and easy-to-perform test, it can be included in the routine diagnosis of equine piroplasmosis, helping to monitor the disease in places where molecular techniques are not accessible.

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Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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