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Health and epidemiological approaches of *Trypanosoma evansi* and equine infectious anemia virus in naturally infected horses at southern Pantanal

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ABSTRACT

Equine infectious anemia virus (EIAV) and *Trypanossoma evansi* are endemic in Brazilian Pantanal Biome, an important area for livestock production. In this sense, we evaluated the epidemiological single and co-infection effects of *T. evansi* and EIAV in naturally infected horses in the southern Pantanal wetland by serological tests and hematological assays. Both higher seroprevalence and heath poor condition of the sampled animals were associated with differences in horse management between farms. We found that the negative animals for both infectious agents (NN) represented the major group in F1 (37%), and the smallest group in F2 (19%). Furthermore, we recorded higher EIAV seroprevalence (56%) in F2, compared to F1 (38%). We observed that *T. evansi* infection was mostly related to young horses, as seen by their higher seroprevalence, ranging from 70.7% in the beginning of the rainy season to 81% in the end of flood period, in comparison with the values of 42% and 68%, respectively, in working animals. on the other hand, working animals showed a higher seroprevalence for EIAV (48%) in both seasons than young horses. We observed that the management of working horses could be a risk factor of EIAV infection. On the other hand, as *T. evansi* is maintained in the study region by many species of wild mammals, the mechanical transmission through blood-sucking vectors ensures the infection to horses since early. Our results showed that single or co-infection by EIAV and *T. evansi* caused different degree of anemia in the infected animals. Moreover, the health of horses in Brazilian Pantanal is also influenced by differences in horse management and environmental circumstances.

1. Introduction

In classic epidemiology, the clinical presentation of a particular infectious disease is understood as a product of an intricate relationship involving an infectious agent, the host's immune response and environmental factors. However, parasites co-occur widely and the consequences of these interactions are poorly understood (Grenfell and Dobson, 1995; Cox, 2001; Pfaff and Candolfi, 2003; McKay, 2006; Rohani et al., 2008). Indeed, co-infections are an important emerging area of research in health and clinical care (Singer, 2010).

A blood protozoan *Trypanosoma evansi* (Kinetoplastida, Trypanosomatidae) is a monomorphic and extracellular parasite mechanically transmitted by blood-sucking flies (Tabanidae and Stomoxydae) in many tropical and sub-tropical areas around the world (Hoare, 1972; Gardiner and Mahmoud, 1990). In the Brazilian Pantanal, a vast flood plain in the core of South Amarica, *T. evansi* is enzootic, infecting both domestic (dogs, horses, bovines, buffaloes and sheep) and wild animal species (coati, capybara, bats, small rodents, marsupials, armadillos, feral pigs, collared peccary and white-lipped peccary) (Dávila et al., 2003; Herrera et al., 2004, 2005, 2008). When *T. evansi* causes severe disease in horses we can observe severe anemia, immunosuppression, and nervous symptomatology shown by hind-limb paresis (Silva et al., 1995a,b). In the Pantanal, trypanosomiasis by *T. evansi* is locally called "mal de cadeiras" and sick animals are usually treated by diminazene acetu-

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rate (7 mg/kg). Nevertheless, Seidl et al. (2001) reported that about 13% of the horses in this region can be expected to die due to *T. evansi* if no treated.

Equine infectious anemia (EIA) is a disease of considerable importance to equine health due to its immunosuppressive effects and severe anemia that significantly impairs animal performance (Issel and Coggins, 1979; Cheevers and McGuire, 1985; Cook et al., 2013). The EIAV (Retroviridae, Lentivirus) is also mechanically transmitted by large hematophagus insects, including *Stomoxys calcitrans* (stable fly) and *Tabanus* sp. (horse fly) (Baldacchino et al., 2013, 2014). However, transmission assigned to humans by using contaminated needles and other fomites plays an important role in EIAV dissemination (Issel and Foil, 1984; Barros and Foil, 2007). The endemicity of EIAV has been reported in the southeastern and northern Pantanal wetland, with seroprevalences ranging from 24.8% to 31.5% respectively (Silva et al., 1999; Borges et al., 2013).

Although they belong to different taxa, *T. evansi* and EIAV are transmitted mechanically by blood-sucking flies and cause severe anemia and immunosuppression in horses. Due to environmental features, socioeconomic traits, and public policies, these diseases remain endemic in some areas of Brazil, including Pantanal region (Silva et al., 1995b, 1999; Seidl et al., 2001; Borges et al., 2013). Horses are indispensable to the traditional extensive cattle ranching in the Pantanal wetland and the health effects of single or co-infection by *T. evansi* and EIAV are still poorly described. In this sense, we evaluated the risk factors associated with the occurrence of *T. evansi* and EIAV, as well as the health status of naturally single and co-infected horses at the southern Pantanal.

2. Materials and methods

2.1. Study area and collected samples

The Brazilian Pantanal is a large (160,000 km^2) seasonal flood plain, bordering Bolivia and Paraguay. In this biome, four million livestock sustain an important economic activity in extensive cattle raising, sharing the same habitats with abundant wildlife. The most distinctive features of this region are annual alternative periods of flood and dry, that have strongly varied in intensity through the years. The vegetation is characterized by exuberant native grass at summer, the rainy season. However, at the end of summer the grassland becomes flooded and during the drought, it becomes fibrous and water is extremely scarce. This highly seasonal climate affects the behavior, spatial distribution, and body condition of both domestic livestock and wild animals (Pozer and Nogueira, 2004; Junk et al., 2006).

In order to know the *T. evansi* and EIAV seroprevalences in the studied area, and determine whether there were differences between cattle ranches, at the first excursion (January 2012) we collected blood samples from all adult working horses in two distinct farms from the southern Pantanal, 30 km distant from each other: F1 (n = 73) and F2 (n = 32). In F1, the management of working horses consisted in a rotation system using two groups of horses. Additionally, only one group of working horses was in the service at a time. On the other hand, animals in F2 were submitted to the service throughout the year and always kept in a restricted area with low availability of pasture. Moreover, horses in F2 shared fomites and needles. In contrast to F2, animals in F1 were subjected to an annual vaccination program against rabies, encephalitis and influenza, as well as deworming (ivermectin and organophosphate) two times per year.

To evaluate if the hematological parameters of the horses would be influenced by (i) infection with EIAV and *T. evansi* (separately or together), (ii) environmental conditions and (iii) the age of the animals, we collected blood sample from all horses of F1 (n = 108) in two times: beginning of flood season (December 2012) and at the end of the rainy season (April 2013). These horses were divided into two categories: (A) untrained young horses, not in service (12–30 months old) (n = 58); and (B) working tamed animals (over three years old) (n = 50). Working horses (category B) were subjected to more physical effort due to the long distances they roamed (often in flooded areas) and management with cattle. Unlike the untrained animals that are bred dispersed over large areas (over 1,000 ha), working horses remained in restricted places, close to each other, for approximately 40 days, according to the cattle management, favoring the mechanical transmission by blood sucking flies.

Blood was collected via jugular puncture, conditioned in sterile tubes containing anticoagulant (ethylenediamine tetraacetic acid, EDTA), and kept refrigerated for a maximum of six hours to perform parasitological and hematological assays. Serum samples were obtained from blood collected in tubes without anticoagulant and kept frozen at −4 °C until the serologic tests. Blood collection was approved by the Ethics Committee on Animal Use (CEUA) of Universidade Católica Dom Bosco/UCDB, Mato Grosso do Sul, Brazil (registration number 021/2015).

2.2. Laboratory procedures

In the field laboratory, during the first six hours after blood collection, we quantified the Packed Cell Volume (PCV), using a micro-centrifuge, and counted Red Blood Cell (RBC) and White Blood Cell (WBC) in Neubauer chambers. Blood smears for WBC differential count were fixed with methanol and stained with May–Grunwald Giemsa. The mean corpuscular volume (MCV) was calculated from the results obtained in the RBC and PCV values. In this study, we considered the PCV, RBC and MCV as health condition indicators; monocyte and neutrophils counts as indicators of infection responses; and lymphocyte counts as an indicator of immune active response.

The parasitological test for *T. evansi* was performed during the first six hours after blood collection, in duplicate, according to Microhaematocrit Centrifuge Technique (MHCT) described by Woo (1970). The seroprevalence of *T. evansi* infection was detected by the Indirect Fluorescence Antibody Technique (IFAT), according to Camargo (1964). The antigen for *T. evansi* was obtained by ion exchange chromatography on DEAE cellulose column (Lanham and Godfrey, 1970) from cryopreserved *T. evansi* parasited blood expanded in rats immunossupressed with 40 mg/kg of cyclophosphamide three days before inoculation. All animal handling procedures had the permission of the Ethics Committee on Animal Use (CEUA) of the Instituto Oswaldo Cruz/FIOCRUZ, Rio de Janeiro, Brazil (registration number: P0292-06). As previously described by Herrera et al. (2004), the positive control serum of the reaction was coming from the positive animals in parasitological MHCT with titre ≥1/640. Negative controls were obtained from horses bred in Rio de Janeiro, a *T. evansi* free area. The cut off (≥1:40) was considered as the lower titer of serum samples in which parasites could be detected by MHCT.

The seroprevalence of EIAV infection was detected by the Agar Gel Immunodiffusion Test (AGID) according to Coggins et al. (1972). We used a kit commercialized by Bruch Laboratory (http://bruch.com.br/005/07) currently approved by the Brazilian government, obtained by the collaboration of the Laboratory of Veterinary Virus Diseases of the Federal Rural University of Rio de Janeiro (UFRRJ).

2.3. Statistical analysis

The difference between the averages of the hematological values obtained in F1 and F2 and in the two management categories within F1 (untrained young horses and working tamed animals) were tested by the Mann–Whitney test. To investigate the influence

Table 1
Seroprevalence of *T. evansi* and EIAV in different groups of infection in the two sampled farms of the southern Pantanal. NN – negative animals for both *T. evansi* and AIE virus infection; PN – positive animals only for *T. evansi* infection; NP – positive animals only for AIE virus infection; PP – co-infected animals by *T. evansi* and AIE virus. The absolute number is followed by the percentage in parenthesis. Statistical differences at $p < 0.005$ are marked with asterisks.

Groups	F1	F2
NN	27 (37.0)*	6 (18.8)*
NP	12 (16.4)*	9 (28.1)*
PN	18 (24.7)	8 (25.0)
PP	16 (21.9)	9 (28.1)
Total	73	32

Table 2
Seroprevalence of *T. evansi* and EIAV in different groups of infection in F1 farm of the southern Pantanal in two distinct periods. NN – negative animals for both *T. evansi* and AIE virus infection; PN – positive animals only for *T. evansi* infection; NP – positive animals only for AIE virus infection; PP – co-infected animals by *T. evansi* and AIE virus. The absolute number is followed by the percentage in parenthesis. Statistical differences at $p < 0.005$ are marked with asterisks.

Groups	Beginning of the rainy season	End of the flooded season
NN	31 (28.7)*	17 (15.7)*
NP	15 (13.9)*	10 (9.3)*
PN	44 (40.7)	57 (52.8)
PP	18 (16.7)*	24 (22.2)*
Total	108	108

of infections, individually or together, in hematological parameters, we applied the Kruskal–Wallis test variance in the sampled horses arranged as the following:

NN – negative animals for both *T. evansi* and AIE virus infection;
PN – positive animals only for *T. evansi* infection;
NP – positive animals only for AIE virus infection;
PP – co-infected animals by *T. evansi* and AIE virus.

To verify if there was an influence of the seasons on health profile of rearing untrained animals and working tamed animals from F1, we used the Wilcoxon test. The phi coefficient was used to test if there was a correlation between these categories and the presence and absence of infections. To check if there was a significant difference in the prevalence of infection by *T. evansi* between seasons (December and April), we used the Kappa test. All analyzes were performed with the Bioestat Program 5.0 and we considered a significance of 95%.

3. Results

We observed differences in seroprevalence of *T. evansi* and EIAV infections, individually or together, according to the farms. The negative animals for both infectious agents (NN) represented the major group in F1 (37%), and the smallest group in F2 (18.8%) (Table 1). Furthermore, we recorded higher EIAV seroprevalence (56%) (including NP and PP horses) in F2, compared to F1 (38%). In addition, we did not observe patent parasitaemia of *T. evansi* or clinical signs suggestive of either the two infections.

Althought we observed high seroprevalences of horses infected only by *T. evansi* (PN) in beginning of flood season (40.7%) and at the end of the rainy season (52.8%) (Table 2), our results showed a significant difference of *T. evansi* infection between young and working animals in the beginning of the rainy season (December) (Table 3). We recorded the increase of seroprevalence in working horses at the end of the flooded season due to the seroconversion of 19 horses from December 2012 to April 2013, corresponding to 17% of the 108 analyzed animals. No animal showed clinical symptom of tripanosomiasis due to *T. evansi*, and only one young horse presented patent *T. evansi* parasitaemia in December, as detected by MHCT.

Table 3
Seroprevalence of *T. evansi* and EIAV in young and working horses of F1 farm of the southern Pantanal in two distinct periods. The absolute number is followed by the percentage in parenthesis.

	Beginning of the rainy season		p value
	Young horses	Working horses	
T. evansi	41 (70.7)	21 (42.0)	**p = 0.0049**
EIAV	9 (15.5)	24 (48.0)	**p = 0.0006**
	End of the flooded season		
	Young horses	Working horses	
T. evansi	47 (81.0)	34 (68.0)	p = 0.1812
EIAV	10 (17.2)	24 (48.0)	**p = 0.0013**

Table 4
Hematological mean values (averages and standard deviation) between horses from F1 and F2 of the southern Pantanal. of The p-values were obtained by the Mann–Whitney test.

Hematological Values	F1	F2	p-value
PCV (%)	31.8 (± 5.33)	30.6 (± 3.71)	0.3563
RBC ($\times 10^6/\mu L$)	7.19 (± 2.14)	5.6 (± 1.37)	**<0.0001**
MCV (ft)	46.8 (± 12.3)	57.6 (± 13.4)	**0.0004**
WBC ($\times 10^6/\mu L$)	12.343 (± 3.238)	10.237 (± 2.805)	**0.0006**
Lymphocytes ($\times 10^6/\mu L$)	7.928 (± 2.921)	5.016 (± 1.600)	**<0.0001**
Neutrophils ($\times 10^6/\mu L$)	3.594 (± 2.310)	4.192 (± 1.831)	0.1026
Eosinophils ($\times 10^6/\mu L$)	503 (± 402)	552 (± 321)	0.3315
Monocytes ($\times 10^6/\mu L$)	309 (± 259)	449 (± 449)	0.0565

We found that the seroprevalence of horses infected only by EIAV (NP) was smallest in the two periods of sampling (13.9% and 9.3%) (Table 2). Furthermore, the seroprevalence of working horses infected by EIAV was the same in December and April (48%), only one young animal seroconverted (Table 3). Moreover, our results showed a positive correlation between EIAV infection and working horses in both beginning of flood season and at the end of the rainy season.

Our results suggest a better general health profile in F1 horses because the indicators of health condition and immune active response (RBC and lymphocytes counts) were significantly different in horses of F1 than in F2 horses (Table 4).

The influence of infections, individually or together, in hematological parameters, showed that in the beginning of the rainy season, the health conditions expressed by RBC counts and PCV values, in horses at F1 differed statistically among the groups. The infection by EIAV (NP) and co-infection (PP) promoted a significant decrease in RBC averages ($7.10 \times 10^6/\mu L$ and $7,48 \times 10^6/\mu L$ respectively) comparing to horses only infected by *T. evansi* (PN) and non-infected (NN) ($8.84 \times 10^6/\mu L$ and $8.18 \times 10^6/\mu L$ respectively) ($p \leq 0.02$). However, horses infected only by *T. evansi* (PN) showed lower average values of PCV (29%).

We also observed that the health of NP and PP horses at the end of the flooded season (April 2013) was severely affected. In fact, in this period, the PCV values of horses infected only by EIAV (NP) and co-infected (PP) horses (33.3% and 35% respectively) was smaller than NN (39.6%) and PN (37.6%) groups ($p < 0.02$). Moreover, the RBC mean of NP horses ($7.19 \times 10^6/\mu L$) was lower than in the PN group ($9.10 \times 10^6/\mu L$) ($p < 0.05$), as well as, the RBC mean of the PP group ($7.22 \times 10^6/\mu L$) was lower than the PN and NN ($8.48 \times 10^6/\mu L$) groups ($p = 0.05$). Furthermore, NP horses presented a significant higher infection response, expressed by neutrophils mean ($8411/\mu L$), than the PN ($7917/\mu L$) and PP group ($6580/\mu L$) ($p < 0.01$).

In addition, we observed that young animals present means of PCV, RBC, WBC and lymphocyte significantly higher, and MCV significantly lower, than the working animals, in the beginning and

Table 5
Hematological values (averages and standard deviation) of young untrained (n = 58) and working horses (n = 50) of the southern Pantanal region. The p-values were obtained by the Mann–Whitney test.

Hematological values	Beginning of the rainy season			End of the flooded season		
	young untrained	working horses	p-value	young untrained	working horses	p-value
PCV (%)	38.8 (±3.3)	34.4 (±5.4)	**<0.0001**	38.3 (±3.7)	35.3 (±6.1)	**0.0025**
RBC ($\times 10^6/\mu L$)	8.73 (±1.29)	7.18 (±1.53)	**<0.0001**	8.87 (±1.51)	7.05 (±1.68)	**<0.0001**
MCV (ft)	45.2 (±6.2)	48.8 (±6.1)	**0.0027**	44.3 (±7.7)	51.7 (±10.8)	**<0.0001**
WBC ($\times 10^6/\mu L$)	14.366 (±4.536)	12.336 (±2.522)	**0.0015**	17.984 (±6.212)	11.848 (±3.275)	**<0.0001**
Lymphocytes ($\times 10^6/\mu L$)	8.563 (±3.520)	6.292 (±2.559)	**<0.0001**	8.019 (±3.972)	3.965 (±1.747)	**<0.0001**
Neutrophils ($\times 10^6/\mu L$)	4.660 (±2.251)	4.968 (±2.380)	0.5962	7.748 (±4.634)	6.410 (±1.822)	**0.0012**
Eosinophils ($\times 10^6/\mu L$)	620 (±432)	628 (±387)	0.9044	775 (±687)	646 (±355)	0.8198
Monocytes ($\times 10^6/\mu L$)	524 (±459)	448 (±409)	0.2782	1.584 (±1.293)	788 (±741)	**<0.0001**

in the end of the rainy season. However, neutrophils and monocytes counts (indicators of infection response) were significantly higher in young animals than the working animals at the end of the flooding period (Table 5).

4. Discussion

In relation to animal health, the differences in hematological parameters between rearing young animals and working horses are expected. In this sense, as observed in this study, young animals commonly have the health condition indicators (PCV and RBC) and active immune response (lymphocytes) higher than working animals (Feldman et al., 2000; Ribeiro et al., 2008). However, the infection response indicators (neutrophils and monocytes) were significantly higher at the end of flooded period and can be associated with environmental stress because grasslands are flooded, concentrating the animals and decreasing the availability of native forages (Pozer and Nogueira, 2004). These factors contribute to reduce the physical condition of the horses, which predispose to parasitic infectious agents (Nelson and Demas, 1996; Tompkins and Begon, 1999). The high counts of monocytes and neutrophils recorded at the end of the flooded period, even in the NN group (horses without *T. evansi* and EIAV infection), indicated that other agents recorded in the studied area as equine encephalitis (Pauvolid-Corrêa et al., 2014), influenza (Gaíva e Silva et al., 2014), Rickettsias (Nieri-Bastos et al., 2014), and *Leptospira* spp. (Jorge et al., 2011) could also be affecting the health of horses.

Our results showed that co-infection by *T. evansi* and EIAV can aggravate the health condition of studied horses, as recorded by low RBC and PCV mean values of PP group in the beginning of rainy season and in the end of flood season. Indeed, the "protozoan/retrovirus" associations in different species have shown that the health of hosts may become compromised (Da-Cruz et al., 1992; Alvar et al., 1997; Sartori et al., 2002; Silveira et al., 2013). However, as previously discussed, the presence of differents infectious agents in a particular host not always constitutes a risk to your health (Tshikuka et al., 1996; Rohani et al., 2008).

The differences between the prevalence of infections and co-infections observed between farms were probably a consequence of the management to which horses were subjected, exemplified by the largest prevalence of negative animals for both infections (NN) in F1 farm (37%), while the NN animals were the lowest in F2 farm (19%). The lack of animal handling and care, exemplified by sharing fomites and low availability of pasture in F2, possibly also reflected in the difference of EIAV seroprevalence (F1 = 38%, and F2 = 56%).

Additionally, the difference in management observed between farms has greater influence on the health status than the infections. We found a better health profile in the F1 horses, as the average values of RBC, WBC, and lymphocytes were significantly higher in F1 than in F2. On the other hand, the poor physical condition observed in F2 animals was the determinant factor that influenced the health profile (observed in hematological parameters) between infected and uninfected horses, including the NN group.

Although we have not found patent *T. evansi* parasitaemia or clinical signs suggestive of either of the two infections, the influence of *T. evansi* on the health condition was evidenced by lower values of PCV and RBC, as shown in the NP group in the end of flooded season. In fact, horses infected by *T. evansi* can present subclinical infections with mild anemia in this area (Herrera et al., 2004). Actually, alternation between the "epizootic clinical" and "inter-epizootic subclinical" phases (characterized by very low parasitaemias) on a temporal scale is an epidemiological characteristic reported on endemic areas for salivarian trypanosomes (Desquesnes, 2004). As we found lower health condition indicators on the end of flooded season, the environmental circumstances should not be ruled out as a risk factor on the health of animals. Indeed, the clinical disease is an eventual outcome of the parasite–host–environment association (Araújo et al., 2003). Furthermore, EIAV similarly has also influenced the health condition in the studied horses since the animals infected by the virus (NP and PP) showed lower RBC and PCV values in December and April respectively. The etiology and pathogenesis of anemia is complex and cannot be accurately represented by one simple mechanism, and in most cases it is due to two or more causative agents rather than a single one (Távora et al., 2015).

Our results showed that horses are exposed to mechanical transmission by horseflies since birth, as observed by the highest seroprevalences of *T. evansi* infection in young horses: 70.7% and 81% in beginning of the raining season and the end of flooded season respectively. Really, *T. evansi* is maintained by a wide variety of species of wild and domestic hosts in the Pantanal (Nunes and Oshiro, 1990; Silva et al., 1995a; Herrera et al., 2007, 2008). Moreover, during the summer, there was a great occurrence of vectors (Barros and Foil, 1999) and the animals were concentrated to non-flooded areas, favoring the vector transmission. This enzootic character may have strongly influenced the seroconversion of 19 horses from December 2012 to April 2013, supplementing that the flooded period represents a risk factor for *T. evansi* infection, as previously reported (Silva et al., 1995a; Herrera et al., 2004).

Concerning EIAV, since only one animal seroconverted during the summer, period in which the highest density of horseflies has been reported (Barros, 2001; Barros and Foil, 2007), we predict that the risk factor of EIAV transmission would be more associated to the different farms management than to blood-sucking flies. In this sense, our results together with Borges et al. (2013) showed that the EIAV transmission in different regions of Pantanal is strongly associated with human activities.

An important point that should not be ruled out in the health status of infected animals refers to the seasonal weather in the Pantanal wetland that significantly changes the landscape: the dynamics of floods and droughts directly influences the nutritional and immune status of horses. These ecological relationships are not static but dynamic in time and space (Vannier-Santos and Lenzi, 2011). Consequently, it is not expected an epidemiological pattern of a given infectious, with re-emergence of severe outbreaks at irregular time intervals, as previously registered (Rwambo et al., 1990; Silva et al., 1995b; Osório et al., 2008). In addition, as men-

tioned, the co-occurrence of other infectious agents may result in situations where *T. evansi* and EIAV, alternatively, influence the health of the horses. Considering the importance of horse breeding as an essential in the Pantanal cattle ranches, we suggest that the management of horses from Pantanal region should be include nutritional and sanitary care. In the case of horses infected by *T. evansi*, animals must be treated. Related to EIAV, as there is no treatment for retrovirus, and seropositive animals are source of infection, they must be removed.

Conflict of interest

None of the authors have any conflict of interests in this manuscript.

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