

Serologic evidence of equine granulocytic anaplasmosis in horses from central West Brazil

Evidência sorológica de anaplasmose granulocítica equina em equinos do Centro-oeste Brasil

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Received May 18, 2009

Accepted November 9, 2009

Abstract

Ehrlichiosis is a zoonotic disease caused by gram-negative and intracellular obligatory bacterial organisms. Equine Granulocytic Anaplasmosis – EGA (formerly Equine Granulocytic Ehrlichiosis, EGE) is a seasonal disease, normally self-limited in horses. There are few reports in Brazil about this ehrlichial agent, as well as its natural vectors. Nowadays, veterinarians are considering the suspicion of EGA in horses with suggestive symptoms of ehrlichiosis and which do not respond to piroplasmosis treatment. The aim of the present study was to identify horses exposed to the agent *A. phagocytophilum* by serological and molecular techniques. Twenty equine blood and serum samples from the central West region of Brazil were evaluated by microscopic examination of buffy coat smear, enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and nested polymerase chain reaction (nPCR). Additionally, the serodiagnosis of *Theileria equi* by IFA and ELISA were carried out, as well as molecular diagnosis by nPCR. Thirteen (65%) serum samples were positive for *A. phagocytophilum* by ELISA, but none of them were positive by buffy-coat smear examination or nPCR. Antibodies IgG anti-*T. equi* were detected in 18 (90%) and 17 (85%) horses by IFA and ELISA, respectively and the agent was detected in 9 (45%) animals by nPCR. Our data may be considered as important information to understanding the occurrence of EGA and equine piroplasmosis in central West Brazil.

Keywords: Equine granulocytic anaplasmosis, *Theileria equi*, nPCR, ELISA, IFA.

Resumo

A Erliquiose é uma doença zoonótica causada por bactérias gram-negativas e intracelulares obrigatórias. A Anaplasmose Granulocítica Equina – AGE (anteriormente denominada Erliquiose Granulocítica Equina, EGE) é uma enfermidade sazonal, normalmente auto-limitante em equinos. No Brasil, existem poucos relatos deste agente erliquial, bem como de seus vetores naturais. Atualmente, veterinários têm levantado a suspeita de casos de AGE em equinos com sinais clínicos sugestivos de erliquiose e não responsivos ao tratamento para a piroplasmose equina. O objetivo do presente estudo foi identificar equinos expostos a *A. phagocytophilum* por meio de técnicas sorológicas e moleculares. Vinte amostras de sangue e soro de equinos da região Centro-oeste do Brasil foram avaliados por meio do exame microscópico de capa leucocitária, ensaio imunoenzimático indireto (ELISA), reação de imunofluorescência indireta (RIFI) e reação em cadeia da polimerase (nested PCR). Adicionalmente, o diagnóstico sorológico de *Theileria equi* pela RIFI e ELISA foram realizados, assim como o diagnóstico molecular pelo nPCR. Treze (65%) amostras de soro foram positivas para *A. phagocytophilum* pelo teste de ELISA, entretanto nenhum equino foi positivo pelo exame microscópico da capa leucocitária ou nPCR. Anticorpos IgG anti-*T. equi* foram detectados em 18 (90%) e 17 (85%) equinos pela RIFI e ELISA, respectivamente e o agente foi detectado em 9 (45%) animais pelo nPCR. Estes dados sugerem importante informação para o entendimento da ocorrência da AGE e piroplasmose equina no Centro-oeste do Brasil.

Palavras-chave: Anaplasmose granulocítica equina, *Theileria equi*, nPCR, ELISA, IFA.

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Introduction

The Family Anaplasmataceae has four genera – *Ehrlichia*, *Anaplasma*, *Neorickettsia* and *Wolbachia* – which include small obligatory intracellular bacteria with unique host cell specificities (DUMLER et al., 2001; RIKIHISA, 1991; 2003). Horses can be naturally infected by the ehrlichial agent *Anaplasma phagocytophilum* (formerly *E. equi*) causing the Equine Granulocytic Anaplasmosis (EGA) that is a seasonal disease of horses, first reported in 1969 in California (STANNARD et al., 1969; GRIBBLE, 1969). Since then, it has been described worldwide. This agent was found first in humans in 1994 in the United States (BAKKEN et al., 1994) and 16S rRNA gene sequence of *A. phagocytophilum* described in horses and dogs seems to be identical to the *A. phagocytophilum* strain that infects humans (JOHANSSON et al., 1995) and described in many countries including Chile (LÓPEZ et al., 2003), Italy (RUSCIO; CINCO, 2003) and Korea (HEO et al., 2002). The vectors are *Ixodes* ticks (PANCHOLI et al., 1995). *A. phagocytophilum* is presented in various tick-rodent cycles and wild reservoirs in nature (FOLEY et al., 2004). The clinical signs of *A. phagocytophilum* in horses include high fever, depression, partial anorexia, limboedema, petechiae, icterus, ataxia and a reluctance to move (MADIGAN; GRIBBLE, 1987). However, clinical signs are not specific for the disease and it has been posed that different hosts demonstrate several degrees of susceptibility to regional strains of *A. phagocytophilum*. In South America a few cases of human ehrlichiosis are described due to *E. canis* and *E. chaffeensis* in Venezuela (PEREZ et al., 2006); and serologic evidence of human ehrlichiosis is found in Chile and Argentina (RIPOLL et al., 1999; LÓPEZ et al., 2003). In Brazil, suspected cases of human were demonstrated with clinical and laboratory findings and with positive serology for human granulocytic anaplasmosis (HGA) by Calic et al. (2004). However, Brazilian spotted fever (BSF) is the most common form of human *Rickettsia* infection in our country. *Ehrlichia chaffeensis* was detected for the first time in blood samples from Brazilian marsh deer (*Blastocerus dichotomus*) captured in the marshes of the Parana River in Southeast Brasil in 1998 (MACHADO et al., 2006), but no human case have been diagnosed to the present time.

Therefore, general diagnosis is based on association of clinical signs in horses, with seasonality and geographic location associated with characteristic gram-negative inclusion bodies/morulae in the cytoplasm of circulating neutrophils in blood smears (MADIGAN, 1993). And the presence of the vector *Ixodes* ticks is important in the acute phase. The acute phase of the disease can be diagnosed directly by microscopic identification of inclusion bodies in peripheral granulocytes, polymerase chain reaction (PCR) and blood culture (BAKKEN et al., 1994; GOODMAN et al., 1996). PCR methods have been shown to be effective for the diagnosis of acute granulocytic ehrlichial infections (MASSUNG; SLATER, 2003). Antibody detection is also used to support the clinical diagnosis (AGUERO-ROSENFIELD et al., 2000). Indirect immunofluorescent antibody assay (IFA) is the most commonly test used in the laboratory to confirm a diagnosis of *A. phagocytophilum* infection in humans (Human granulocytic ehrlichiosis, HGE). In horses, a differential diagnostic approach

should be made because of such other diseases as encephalitis, liver disease, purpura hemorrhagica, and equine infectious anemia, which can have similar clinical conditions (MADIGAN, 1993). The present study reports the first serologic evidence of granulocytic ehrlichiosis in horses from Brazil. And considering that cases of equine ehrlichiosis can be due to *Neorickettsia risticii*, a screening PCR for the Genus was performed. Additionally, the serodiagnosis and molecular detection of *Theileria equi* were carried out once equine piroplasmosis became widespread and a cause of serious concern in Brazil.

Material and Methods

1. Horses

Twenty cases suspected of equine granulocytic ehrlichiosis were selected from horses with clinical signs suggestive of *A. phagocytophilum* infection. Nineteen mixed-breed horses came from from the Brazilian Army (1º Regimento da Cavalaria de Guarda, Dragões da Independência, Brasilia - DF, Brazil) and one horse from Goiânia County - GO, Brazil. At the time of initial examination the clinical signs were fever, anorexia, lethargy, pale mucous membranes and edema of the limbs. Tick exposure or tick infestation were commonly observed in all horses.

2. Samples

Whole blood was collected aseptically via jugular venipuncture and an aliquot was allowed to clot, then centrifuged and serum was separated. Another part of the sample was EDTA-whole blood that was used as a source for buffy coat smears stained with Giemsa. The remaining blood was stored at -20 °C until assayed.

3. *A. phagocytophilum* serology

An enzyme-linked immunosorbent assay (ELISA) for detection of IgG antibodies against *A. phagocytophilum* was performed (*Ehrlichia equi* IgG antibody Kit – ELISA – Helica Biosystems, Inc.). Briefly, equine serum samples were diluted to 1:100 and allowed to react with *E. equi* / (*A. phagocytophilum*) whole antigens coated on specially treated micro-well plates provided with the kit. The reactions were performed according to the manufacturer's instructions and the results were based on color intensity (absorbance) developed with an optical density (OD) of 450 nm. The visual determinations were made with aid of the color chart provided by the kit. ODs over 0.32 were interpreted as positive.

4. *Theileria equi* serology

Indirect immunofluorescent antibody assays (IFA) were performed to detect IgG antibodies against *T. equi* as previously described by Machado et al. (1994), with a serum dilution of 1: 80. Also, an ELISA test was performed as reported elsewhere (BALDANI et al., 2004) with an antigen concentration of 10 µg.mL⁻¹ and a serum

dilution of 1:100. The immunological activity of each serum was calculated by determining the sample-to-positive serum ratio (S/P), considering positive and negative sera as reference. Horses were considered positives when S/P values at an optical density of 405 nm were equal to or greater than 0.278.

5. DNA isolation

DNA was extracted from 200 µL of EDTA whole-blood EDTA samples using the QIAamp DNA Blood Mini Kit (QIAGEN™), according to the manufacturer's instructions. Samples from the thirteen horses tested as serologically positive ($OD \geq 0.32$) for *A. phagocytophilum* antibodies by the ELISA Kit were analysed. These horse blood samples ($n = 13$) were then collected before (rest, $n = 13$) and after exercise (stress, $n = 13$), and were submitted to buffy coat smear preparations stained with Giemsa for microscopic examination, and to PCR amplifications for *A. phagocytophilum* and *Ehrlichia* spp. DNA extraction was also performed for the detection of *T. equi* by nPCR.

6. PCR amplification

Each extracted DNA sample was used as a template to amplify a specific amplicon in 50 µL reaction mixtures containing 10x PCR buffer, MgCl₂, deoxynucleotide triphosphate (dNTPs) mixture, and DNA Taq polymerase (Invitrogen™), and were carried out with the following specific primers for *Ehrlichia* spp.: ECC (5'-aga acg aac gct ggc ggc aag cc - 3') and ECB (5'-cgt att acc gcg gct gct ggc - 3') that amplify a fragment of 478 bp (PERSING, 1996; KOCAN et al., 2000), for the *A. phagocytophilum* 16sRNA gene: gE3a (5' - cac atg caa gtc gaa cgg att att c - 3'), gE10R (5' - ttc cgt taa gaa gga tct aat ctc c - 3'), gE2 (5' - ggc agt att aaa agc agc tcc agg - 3') and gE9F (5' - aac gga tta ttc ttt ata gct tgc t - 3') that amplify fragments of 932 and 546 bp (MASSUNG et al., 1998), respectively, and for the *A. phagocytophilum* msp2 gene: MSP 465F (5' - tga tgt tgt tac tgg aca ga - 3') and MSP 980R (5' - cac cta acc ttc ata aga a - 3') that amplifies a 550 bp fragment (CASPERSEN et al., 2002). All the amplification reaction mixtures and the cycling parameters were performed as previously described by the authors cited above. The first (PCR) and nested amplifications (nPCR) were performed in a Gradient cycler model PT-200 (Perkin-Elmer™). In each set of amplifications, both positive and negative controls were included. Amplicons were visualized in 1.5% Agarose gels stained with Ethidium Bromide under UV-light. All the steps were carried out in separate rooms to minimize contamination.

The nPCR for the diagnosis of *T. equi* were performed as previously described by Baldani et al. (2008).

Results

1. Clinical signs and presence of ticks

The most common clinical signs found in all tested horses were anemia (pale mucous membranes), fever, anorexia, lethargy and limbs edema. *Amblyomma cajennense* and *Amblyomma* spp. were the tick species found in these animals.

2. Buffy coat smears

None of the samples examined showed any evidence of Anaplasmataceae infection as inclusion bodies and/or morulae structures.

3. *Anaplasma phagocytophilum* serology

Thirteen (65%) of 20 equine serum samples included in this study showed positive reactions ($OD \geq 0.32$) by the ELISA Kit (Helica™). These 13 positive samples were then collected again (after exercise), as previously described, and tested by PCR and nPCR in two sets: before exercise ($n = 13$) and after exercise ($n = 13$).

4. Anaplasmataceae agents PCR and nPCR

None of the blood samples tested ($n = 26$) were positive by PCR amplification using primers ECC/ECB for *Ehrlichia* spp. Also, the amplifications PCR and nPCR for the *A. phagocytophilum* 16sRNA gene (Figure 1) and msp2 gene were all negative. All the results are summarized in Table 1.

5. *Theileria equi* serology and nPCR

Eighteen (90%) equine serum samples tested were positive by the IFA test (cut off titer ≥ 80) and 19 (95%) by the ELISA assay for the same agent. *T. equi* was detected by nPCR in 9 (45%) horses.

Discussion

In the present study none of the twenty equines with clinical signs demonstrated granulocitic morulae or initial bodies in Giemsa blood smears. Clinical anaplasmosis in horses is probably still underdiagnosed in Brazil, especially those horses taking part in sports and traveling to others regions outside of the country where the disease is endemic. On the other hand, clinical anaplasmosis is similar to those caused by infections with other pathogens such as *Babesia caballi*, *Theileria equi*, equine infectious anaemia virus, equine herpesvirus, equine arteritis virus and *Leptospiraceae*. Recently, in Brazil, a spontaneous outbreak of Equine Monocytic Ehrlichiosis (EME) was described, caused by *Neorickettsia risticii*, affecting horses 1-5 years old in the State of Rio Grande do Sul. The most important clinical sign was profuse acute diarrhea, accompanied by anorexia and depression (COIMBRA et al., 2006).

Acute equine ehrlichiosis is evaluated observing the characteristic inclusion bodies in blood smears as well as clinical signs, but chronic cases are not clinically detected. Also, in acute disease a differential diagnosis pattern should be performed because of the nonspecific clinical signs observed. Diseases such as encephalitis, liver disease, purpura hemorrhagica, equine infectious anemia and equine viral arteritis should be ruled out (MADIGAN; GRIBBLE, 1987). The severity of clinical signs of EGA is a function of the age of the horse and the duration of the illness (MADIGAN, 1993). This

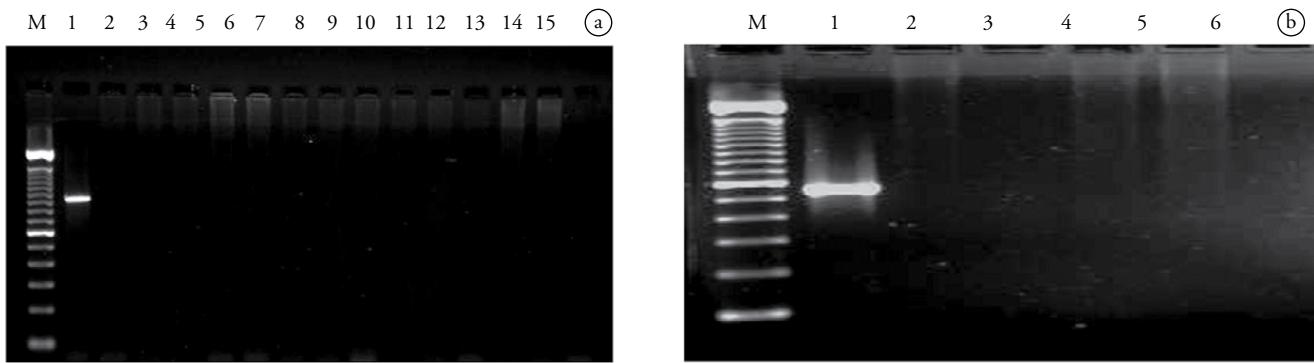


Figure 1. 1,5 % Agarose gel electrophoresis stained with Ethidium Bromide, showing results of equine blood samples tested for *A. phagocytophila* 16SrRNA gene nested PCR (a, b) products obtained after amplification with primer pairs gE3a/gE10R; gE2/gE9F (amplicons size 932 and 556 bp), respectively. a) Lane M: molecular standard size (100 bp); Lane 1, positive control; Lane 2 through 14: horse blood samples and Lane 15, negative control. b) Lane M: molecular standard size; Lane 1, positive control; Lane 2 through 5: horse blood samples and Lane 6, negative control.

Table 1. Results from Buffy coat smear examination, *Babesia equi* Serology (ELISA and IFA), *A. phagocytophilum* Serology (ELISA Kit), and PCR/nPCR (16S rRNA gene), PCR (msp2 gene) and *Ehrlichia* spp. PCR (16S rRNA gene).

Horse Sample	Buffy coat smear	<i>Babesia equi</i>		<i>Anaplasma phagocytophilum</i>			<i>Ehrlichia</i> spp.	
		ELISA (OD)	IFA	ELISA kit	PCR/ nPCR 16S rRNA gene	PCR msp2 gene	PCR 16S rRNA gene	
1	N	N (0.175)	N	P	N	N	N	N
2	N	P (1.207)	P	P	N	N	N	N
3	N	P (0.952)	P	P	N	N	N	N
4	N	P (1.082)	P	P	N	N	N	N
5	N	P (0.919)	P	P	N	N	N	N
6	N	P (1.620)	P	P	N	N	N	N
7	N	P (0.859)	P	P	N	N	N	N
8	N	P (0.784)	P	P	N	N	N	N
9	N	P (0.909)	P	P	N	N	N	N
10	N	P (1.311)	P	P	N	N	N	N
11	N	N (0.225)	N	P	N	N	N	N
12	N	P (1.131)	P	P	N	N	N	N
13	N	P (0.839)	P	P	N	N	N	N
14	NT	P (0.998)	P	N	NT	NT	NT	NT
15	NT	N (0.148)	P	N	NT	NT	NT	NT
16	NT	P (0.901)	P	N	NT	NT	NT	NT
17	NT	P (0.775)	P	N	NT	NT	NT	NT
18	NT	P (2.895)	P	N	NT	NT	NT	NT
19	NT	P (1.323)	P	N	NT	NT	NT	NT
20	NT	P (0.754)	P	N	NT	NT	NT	NT

P = positive, N= negative, NT= not tested, OD = optical density.

can make clinical recognition of EGA difficult at the time of the first examination (MADIGAN; GRIBBLE, 1987).

Serum antibodies have been detected by the ELISA Kit (Helica™) in thirteen of 20 equines (65%) to *A. phagocytophilum*. The most common serologic diagnostic assay is the detection of serum antibodies by IFA, but the ELISA test has been used to evaluate the response of antibodies to *A. phagocytophilum* in horses (MADIGAN et al., 1990; CORSTVET et al., 1993; MAGNARELLI et al., 2000; 2001; LEBLOND et al., 2005; HANSEN et al., 2010). Also, the IFA tests are a useful tool for

diagnosis of EGA convalescence patients (WALLS et al., 1999). Although antibody detection may be of limited value for early serologic diagnosis (van ANDEL et al. 1998), strong serological cross-reactions between all members of the *A. phagocytophilum* have been communicated (DUMLER et al., 1995). Recently, Spolidorio et al. (2010) showed that 25.9% horses from the State of Espírito Santo, Brazil were reactive to at least one of the six *Rickettsia* species tested, and in six horses PCR *Theileria* were detected. It is important to note that no ticks of *Ixodes ricinus*, a common transmitter of EGA, have been described in Brazil.

None of the tested animals showed positive reaction in PCR assays to *A. phagocytophilum*, for either the 16s rRNA gene or *msp2* gene. However, positive nPCR in nine animals to *T. equi* confirmed the presence of the agent by molecular detection. In addition, 90% of all animals tested were positive by the IFA test and 95% by the ELISA assay for the same agent. Previous studies in our laboratory have not confirmed the presence of *A. phagocytophilum* in blood samples tested from two hundred horses with clinical signs of babesiosis or suspected of anaplasmosis by PCR or nPCR (data not shown). In Brazil, the prevalence of equine piroplasmosis is of serious concern, considered as an endemic disease. To detect anti-*T. equi* antibodies, Heuchert et al. (1999) tested 752 horse serum samples from São Paulo State, by IFA and a complementary fixation test (CFT), and reported prevalence rates of 29.6 and 17.6%, respectively. Serum samples from horses from different regions of Brazil were tested by CTF and the results demonstrated the prevalence of *T. equi* infections were 42.48% (KERBER et al., 1999). Also Xuan et al. (2001) examined serum samples from horses in the State of São Paulo and Mato Grosso do Sul, Brazil, and showed that the positive rate for *T. equi* was 81%. Baldani et al. (2004) demonstrated that 75% (67/90) of the horses from the state of São Paulo presented anti-*T. equi* antibodies by IFA and ELISA tests. The prevalence of equine piroplasmosis reported in the present study is higher and may be due to differences in serological tests used, horse breeding operations, intensity of tick infestation or even number of serum samples examined. This study is the first report on the serological evidence of co-infection with Equine Granulocytic Ehrlichiosis and equine piroplasmosis agents in horses from the Brazilian Army and Goiânia county. *Amblyomma cajennense* and *Amblyomma* spp. were the most common tick species found in our animals in this study.

Our data may be considered as important information contributing to understanding the occurrence of these diseases in centralwest Brazil.

Acknowledgments

We express appreciation to Dr. J. Stephen Dumler for *Anaplasma phagocytophilum* DNA-positive controls.

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