

**SEROLOGICAL EVIDENCE OF EXPOSURE TO *Anaplasma phagocytophilum*  
IN HORSES FROM THE RIO DE JANEIRO STATE MOUNTED POLICE  
BRED IN THE URBAN ZONE**

***EVIDÊNCIAS SOROLÓGICAS DA EXPOSIÇÃO À *Anaplasma phagocytophilum*  
EM CAVALOS DA POLÍCIA MONTADA DO ESTADO DO RIO DE JANEIRO -  
BRASIL CRIADOS EM ÁREAS URBANAS***

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**Abstract**

*Anaplasma phagocytophilum* is an emergent hemoparasite in regions where ticks are regularly found. In order to investigate the rate of anti-*A. phagocytophilum* antibody in horses of the Mounted Police of Rio de Janeiro state and therefore the presence of this agent, 41 horses from the Cavalry Squadron (CS) and 50 from the Regiment of Mounted Police (RMP) were selected. For the serologic diagnosis the Indirect Fluorescent Antibody test was performed. In the present work, among 91 equine sera samples anti-*A. phagocytophilum* antibodies, with titer  $\geq 1:80$ , were detected in 11 animals (12%), being five (5%) from males and six (7%) females, with no statistical difference. The adult animals with ages varying between five and 14 years presented the highest rate of positive reaction, although antibodies were detected in animals of all ages. We did not observe statistic differences in relation to the presence of anti-*A. phagocytophilum* antibodies among the animals bred in the CS and RMP. The presence of anti-*A. phagocytophilum* antibodies in horses of the Mounted Police with no clinical signs is indicative that the parasite is present in the enzootic form among the horses of the urban area. The circulation of the parasite among the animals is not dependent of the presence of tick infestations.

**Keywords:** equine; granulocytic anaplasmosis; hemoparasites; IFA; tick.

**Resumo**

*Anaplasma phagocytophilum* é uma hemoparasitose emergente em regiões onde existem carrapatos. Com o objetivo de investigar a frequência de anticorpos anti-*A. phagocytophilum* em cavalos da Polícia Montada do Estado do Rio de Janeiro e, conseqüentemente, os indícios da presença desse

agente em áreas urbanas, foram selecionados 41 cavalos do Esquadrão de Cavalaria (EC) e 50 do Regimento de Polícia Montada (RPM). Para o diagnóstico sorológico, foi utilizado o método de Imunofluorescência Indireta. No presente trabalho, dentre 91 amostras de soro de equinos, foram detectados anticorpos anti-*A. phagocytophilum* com título  $\geq 1:80$  em 11 animais (12%), sendo que cinco (5%) eram machos e seis (7%) fêmeas, sem diferenças estatísticas. Os animais adultos com faixa etária entre cinco e 14 anos foram os que apresentaram maior frequência de reação positiva; no entanto, foram detectados anticorpos em animais de todas as faixas etárias. Não foram observadas diferenças estatísticas quanto à presença de anticorpos anti-*A. phagocytophilum* nos animais criados no EC e no RPM. A presença de anticorpos anti-*A. phagocytophilum* em cavalos da Polícia Montada sem sinais clínicos aparentes é indicativo que *A. phagocytophilum* está presente de forma enzootica na região urbana e a circulação do parasita entre os animais independe da presença de infestações aparentes de carrapatos nos animais.

**Palavras-chave:** anaplasose granulocítica; carrapatos; equino; hemoparasitos; RIFI.

Recebido em: 29 agosto 2012.

Aceito em: 11 dezembro 2014

## Introduction

*Anaplasma phagocytophilum* is an obligate intracellular bacterium of neutrophils in wild and domestic mammals, birds and humans<sup>(1, 2)</sup>. In Brazil, it has been described in horses<sup>(3)</sup>, cervids<sup>(4)</sup>, dogs<sup>(5)</sup>, migratory birds<sup>(6)</sup> and wild carnivores<sup>(7)</sup>.

Even though the disease caused by *A. phagocytophilum*, granulocytic anaplasmosis (GA), is considered a zoonosis, the transmission dynamics of this bacterium, which involve ticks, reservoirs and hosts, have not been extensively studied in Brazil<sup>(4, 7, 8)</sup>, especially in animals that are in close contact with humans, such as horses. GA is characterized by an acute febrile syndrome with clinical and laboratorial characteristics that are common to both humans and horses<sup>(9)</sup>. The clinical form of this condition in horses is associated with poor performance, depression, petechiae in oral, vaginal, nasal and conjunctive mucosae, limb edema and ataxia<sup>(10, 11)</sup>. GA incidence in horses is poorly known and, additionally, infection by *A. phagocytophilum* has been frequently diagnosed as a self-limiting disease with no apparent clinical signs<sup>(10, 12, 13)</sup>. These data highlight the potential role of horses as a reservoir for this bacterium<sup>(14)</sup>. Additionally, the capability of horses to produce anti-*A. phagocytophilum* antibodies that can be detected by commercial tests for up to six months enables the use of this species as sentinels to prevent outbreaks in humans<sup>(15)</sup>.

*Anaplasma phagocytophilum* should be included in the differential diagnosis in cases of suspected equine babesiosis, especially when horses exhibit clinical signs and either the protozoan is not detected in peripheral blood smears or there is no response to chemotherapy<sup>(16)</sup>.

The main *A. phagocytophilum* vectors are ticks of the *Ixodes* genus, which include *I. persulcatus*, *I. ricinus*, *I. pacificus* and *I. scapularis*<sup>(17-20)</sup>. Researchers have identified differences in transmissibility within this genus, which can explain variations not only in GA incidence and severity, but also in the vertebrate hosts that are affected.<sup>(21)</sup> In Central America some tick species were reported as probable vectors of *A. phagocytophilum*, like *Amblyomma cajennense*, *Anocentor nittens* e *Rhipicephalus microti*<sup>(22)</sup>.

Considering that Brazil's Southeastern Region has the largest population of horses in the country due to the intensification of activities that put these animals in close contact with humans, the objectives of this study were to assess the rate of anti-*A. phagocytophilum* antibodies in the horses of the mounted police of Rio de Janeiro state, which are used in activities such as patrolling, classic equestrian and hippotherapy; to determine possible risks of infection by the parasite associated to breedings, and also to draw attention to the importance of the detection of *A. phagocytophilum*.

## Materials and Methods

During January and February of 2010, we collected blood samples of 91 of the 182 horses of the mounted police of Rio de Janeiro state, from which 154 were males and 28 females; 50 samples came from the Mounted Police Regiment (RMP) of Rio de Janeiro state and 41 from the Cavalry Squadron (CS). This investigation was performed in the western region of the metropolitan area of Rio de Janeiro state, southeast of Brazil. Sample size was calculated using the statistical software Epi-info 2000, with expected anti-*A. phagocytophilum* antibodies rate of 17.03% and acceptable up to 25% with confidence interval of 95%.

The horses were raised in a semi-intensive breeding system; they were directly examined by veterinarians, subjected to sanitary handling, tested for equine infectious anemia (EIA), treated for the control of endo- and ectoparasites, and vaccinated against equine influenza (EI), equine herpesvirus Type-1 (EH-1), equine encephalomyelitis, tetanus, and rabies. Blood smears examination for hematozoa was performed in association to hemogram as a protocol for animals with reduced performance, apathy or fever syndrome. Blood samples were obtained from jugular vein punctures with needles and adapters for vacutainer tubes; samples were later centrifuged for serum extraction. Serum samples were stored in duplicates in plastic 2.0 ml eppendorf tubes and kept at -20 °C. For serological diagnosis, the indirect fluorescent antibody test (IFA) method was used. Specifically, samples were tested with the *Anaplasma phagocytophilum* IFA Equine Antibody Kit (Fuller Laboratories©, Fullerton, California, USA). Serum samples were diluted 1:80 in phosphate buffered saline solution (PBS) with a pH of 7.2 in the proportion of 10 µL of sample for 790 µL of PBS. 10 µL of the diluted samples were pipetted in each well of slides containing antigenic substrate for *A. phagocytophilum* (HL60 cells containing *A. phagocytophilum* morulae), reserving two wells for the addition of positive and negative control samples containing, respectively, reactive equine serum diluted from 1:80 to 1:640 and non-reactive equine serum diluted at 1:80. The slides were incubated at 37 °C for 60 minutes in a humid chamber, washed in PBS, and then dried. Next, we added 10 µL of conjugate (equine anti-IgG isothiocyanate marked with fluorescein to each well (Fuller Laboratories©, Fullerton, California, USA). The slides were incubated for 60 minutes at 37 °C in a moist room. After three washings with PBS and drying, slides were mounted with 50% glycerol diluted in PBS. Results were visualized by examining the slides using a Zeiss immunofluorescence microscope (AXIOSHOOP 40) with a Carl Zeiss digital camera (AXIO CAM MRC – HAL100) for image capture that was coupled to a microcomputer with Zeiss AxionVision LE 4.8.2 software. Positive samples reacted at the dilution of 1:80; this titer was the cut-off and allowed the observation of fluorescence in the morulae (Figure 1).

Additionally, we carried out an epidemiological investigation, observing aspects associated to animal identification (breed, age and sex), animal origin, work type, environment, nutritional and

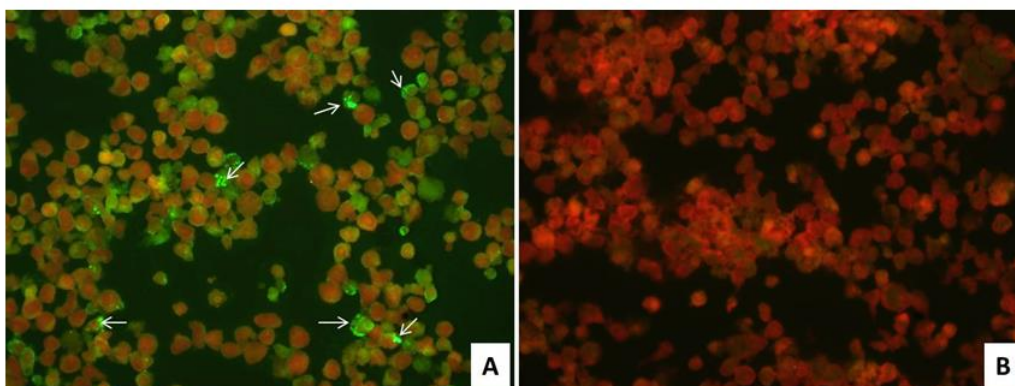
sanitary handling, breeding system, transit, participation in events, animal commercialization, reproduction types, history of hemoparasites, presence of ectoparasites and wild animals, and the presence of other species of domestic animals.

From the data collected during the epidemiological investigation we calculated the rate of positive animals associated to the ages. The relative risk for *A. phagocytophilum* infection associated to gender was calculated using Fisher's Exact Test with Katz approximation and to the origin we used the Chi-Square test with Yates correction and Katz approximation. All calculations were made with 95% confidence interval using the software Graphpad Instat.

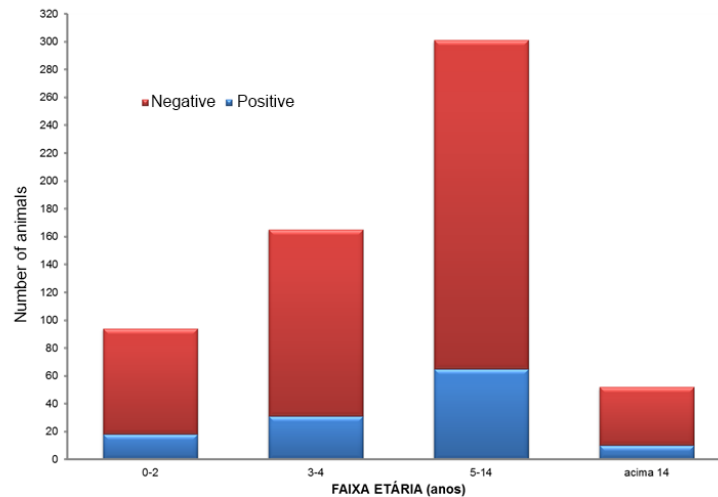
## Results

In the present study, 91 equine serum samples were tested, and anti-*A. phagocytophilum* antibodies were detected in 11 (12%) animals being 1 (1%), 0 (0%), 9 (10%) and 1 (1%) in the range of 0 to 2, 3 to 4, 5 to 14 and over 15 years old, respectively. Although adult animals aged between five and fourteen years had the the highest frequency of positive reactions, antibodies against *A. phagocytophilum* were detected in animals of all ages (Figure 2). There were no statistical differences observed with regard to the presence of anti-*A. phagocytophilum* antibodies between the animals raised at the CS and RMP (Table 1). Of the positive animals, five (5%) were males and six (7%) were females (Table 1); no statistical difference was observed ( $p=0.0868$ ).

The horses of the military police participate in the activities of mounted policing in Rio de Janeiro state, horseback riding throughout Brazil and hippotherapy with special children. Horses in both squadrons presented fifteen cases of babesiosis in the last five years, all diagnosed by blood smear. During this period, two animals that had reduced performance, fever, leucopenia and were suspected for babesiosis and did not respond to specific therapeutic treatment, were tested for the presence of antibodies against *A. phagocytophilum* using IFA. After positive test response, animals were treated with oxytetracycline hydrochloride (10 mg/kg) and recovered after three days.



**Figure 1:** Indirect Fluorescent antibody test (IFA) in sera of horses of the Rio de Janeiro State Military Police, Brazil. In **A**, arrows point to fluorescent morulae in positive samples, contrasting with a negative reaction observed in **B**.



**Figure 2:** Rates of seropositive and negative horses for antibodies anti-*A. phagocytophilum* antibodies in breedings of the Mounted Police of Rio de Janeiro state, Brazil, according to age ranges 0 to 2 years old, (1%, 1%), 3 to 4 (0.0%, 1%), 5 to 14 (10%, 70%) and over 15 (1%, 16%) compared by Chi-square test ( $p= 0,8828$ ), respectively.

**Table 1:** Relative risk for the presence of anti-*Anaplasma phagocytophilum* antibodies associated to sex and origin of the horses of the Cavalry Squadron (CS) and Regiment of Mounted Police (RMP) of Rio de Janeiro State, Brazil

Variables	Horses		Total	P-value	Relative risk (Rr)	Confidence interval (95%)
	Positive	Negative				
CS	8 (9%)	33 (36%)	41 (45%)	0.0596 <sup>a</sup>	3.252 <sup>b</sup>	0.9212
RMP	3 (3%)	47 (52%)	50 (55%)			11.480
Males	5 (5%)	58 (64%)	63 (69%)	0.0868 <sup>c</sup>	0.3704 <sup>b</sup>	0.1232
Females	6 (7%)	22 (24%)	28 (31%)			1.113
<b>TOTAL</b>	<b>11 (12%)</b>	<b>80 (88%)</b>	<b>91(100%)</b>			

<sup>a</sup> To Test Chi-Square and With Yates correction.

<sup>b</sup> With Katz approximation.

<sup>c</sup> To Fisher's Exact Test

## Discussion

We observed that 12% of the examined horses of Rio de Janeiro state Mounted Police that were raised at a urban region of the city of Rio de Janeiro tested positive for the presence of anti-*A. phagocytophilum* antibodies. However, with the exception of one animal that had already been serologically tested and treated, there were no clinical signs reported in the epidemiological investigation that could lead to clinical suspicion of the disease. In Brazil, three reports on the presence of anti-*A. phagocytophilum* antibodies in horses have been published. In 1984, MESQUITA NETO et al.<sup>(3)</sup>. reported *Ehrlichia equi* in horses from the municipality of Betim, Minas Gerais state. In 2010, SALVAGNI et al.<sup>(16)</sup> reported 65% seropositivity for *A.*

*phagocytophilum* using an ELISA-based test; however, negative results were reported when PCR was used for pathogen detection. In contrast to the present study, blood samples were collected from equines in military use in the central region of the city of Goiania with clinical symptoms suggestive of infection. Previously, PARRA<sup>(13)</sup> reported that 3% of 250 horses in the southeastern Brazilian state of São Paulo were seropositive by ELISA for anti-*A. phagocytophilum* antibodies, however, all animals were negative for hemoparasites in the PCR test. Thus, EGA is possibly being neglected in Brazil due to the difficulty of conclusive diagnosis and its self-limiting nature. This hypothesis is reinforced if we compare the 12% of seropositive equines observed in this research to the levels reported for horses in some European countries, such as Holland (83.3%) and Denmark (22.3%)<sup>(23)</sup>. High frequencies of *A. phagocytophilum* seropositivity in horses was also reported by Frazen et al.<sup>(12)</sup> and Passamonti et al.<sup>(24)</sup> in Sweden (16.6%) and Italy (17.3%); however, these same authors reported lower seropositive frequencies in animals in France (11.3%) and Spain (6.5%) compared to the horses of the Regiment of the Mounted Police of Rio de Janeiro. In the United States of America (USA), the seroprevalence for *A. phagocytophilum* in horses in eastern cities ranged from 17.6% to 29% in regions where ticks are endemic; seroprevalence was 3.8% in areas where the presence of ticks was insignificant<sup>(25)</sup>. This could be an explanation for the few suspect cases among horses of the Mounted Police of Rio de Janeiro, in which AGE test is applied; these animals are submitted to a rigorous ectoparasite control regimen.

Even with the periodic parasite control on horses of the Mounted Police, one cannot discard the importance of ticks in the epidemiologic chain for the analyzed breedings, as some researchers have verified differences in the tutorial capacity of different species of ticks, identified as possible transmitters, what could explain the ecologic variations and the emerging of granulocytic anaplasmosis in different demographic regions in a way that corroborates with the incidence and gravity of the disease, besides the affected vertebrate hosts<sup>(8,21,26,27)</sup>. These researchers reinforce this hypothesis because in Europe the infection was detected in ruminants, in California in equids, in Asia in humans and not in domestic animals, and in East USA in dogs and humans.

The most efficient transmitter of *A. phagocytophilum* to horses is the adult tick; it is known that nymphs most frequently transmit the pathogen from animals to humans<sup>(25,28)</sup>. Due to the predominantly tropical climate in Rio de Janeiro, all of the stages of the tick life cycle are observed throughout the year on various animals, increasing the probability of horses acquiring hemoparasites.

Samples were collected from horses of the Military Police of Rio de Janeiro during the summer, when conditions are propitious for tick infection; however, observations of ticks in the facilities or on the horses were rare. The principal reservoirs for *A. phagocytophilum* in Europe and USA are wild animals, rodents and deer in addition to the same vectors for domestic animals.<sup>(29,30)</sup> *Anaplasma phagocytophilum* was detected in dogs in Brazil by PCR<sup>(5)</sup>, signs of the presence of the agent in goats and sheeps in the northeastern region were reported by RAMOS et al.<sup>(31)</sup>, besides migrating birds<sup>(6)</sup>, cervids<sup>(4)</sup> and wild carnivores<sup>(7)</sup>. In this epidemiologic investigation we noted the presence of wild animals such as opossums, armadillos, and birds in both analyzed breedings. As there was no difference in these observations for both breedings, these variables were not considered in the risk analysis of the infection by *Anaplasma phagocytophilum*. But one cannot discard the importance of these animals for the infection in equines of the Military Police of Rio de Janeiro. For horses in rural regions, other natural reservoirs may be involved in anaplasmosis transmission, including human infection, which in Brazil is still a rarely studied disease<sup>(6,4)</sup>.

The results concerning the ages (Figure 1) revealed a higher number of seropositive adult animals and fewer young and elderly animals, but other authors<sup>(32)</sup> found significant correlation ( $p \leq 0,03$ ) between age of equids over 11 years old and the presence of anti-*A. phagocytophilum* antibodies. Engvall & Egenvall<sup>(33)</sup> concluded, differently from the results for the Rio de Janeiro Mounted Police, the prevalence increases with the age what turns it a risk factor due to the higher rate of exposition to the tick during life. This age is also the most commonly used for work and sport; therefore, these animals are more often exposed to potential risk factors including journeys, exposure to animals from other regions, and stress<sup>(33,34)</sup>.

In this study we did not verify significant difference for males and females in the seropositivity for anti-*A. phagocytophilum* antibodies, what means sex is not a risk factor for *A. phagocytophilum* infection. This corroborates with the findings of Bullock et al.<sup>(34)</sup> in 733 equids studied in the region of Minnesota and Wisconsin.

There was no significant variation ( $p=0.0596$ ) in the seropositivity for anti-*A. phagocytophilum* antibodies between CS and RMP (Table 1), what can be explained by the free transit of animals between both environments as verified in the epidemiological report.

The origin of animals was not considered a risk factor for infection with *A. phagocytophilum* in horses of the Mounted Police of Rio de Janeiro state; however, the bleach of seropositive animals allows suspecting the existence of possible outbreaks of infection located at the other properties or microregions of the city of Rio de Janeiro.

The occurrence of *A. phagocytophilum* infection in horses from the urban area of the city of Rio de Janeiro, principally those in close quarters with other domestic animals and humans, may suggest a potential risk for emergent zoonoses in Brazil, similar to what has already been observed in the USA and in Europe<sup>(25,35)</sup>. However, this parasitosis should be better investigated, including studies that confirm diagnosis through molecular biology. Molecular-based diagnosis represents a challenge because of the genetic diversity of surface proteins displayed by various strains in accordance with the geographic distribution of *A. phagocytophilum*. Additionally, cross-reactivity with other *Anaplasma* spp. also poses difficulties for molecular identification<sup>(36)</sup>.

In Brazil, equine monocytic ehrlichiosis caused by *Neorickettsia risticii* was described in Rio Grande do Sul, and has its cycle dependent of a trematode and snail as intermediate hosts<sup>(37,38)</sup>. The pathological clinical manifestation, with severe enterocolitis, presents differences when compared to equine granulocytic ehrlichiosis<sup>(39)</sup>. In horses the cross-reaction in serological tests among natural species of *N. risticii* and *A. phagocytophilum* was not yet proved<sup>(1)</sup>. The control of parasites and care taken in the handling of horses of the Mounted Police of Rio de Janeiro made the identification of the species of ticks involved in the transmission of *A. phagocytophilum* to these animals impossible. Although the cases of equines with symptoms and seropositivity in Brazil documented by SALVAGNI et al. in 2010<sup>(16)</sup> presented with histories of infestation by *Amblyomma* spp. we have not found scientific studies demonstrating the importance of these arthropod species in transmission. In 2004, two HGA cases of suspected anaplasmosis in Brazil were described and diagnosed through clinical findings and indirect immunofluorescence assays; however, both individuals were only sero-reactive for *Ehrlichia chaffeensis*<sup>(40)</sup>. HGA is a zoonosis that is found in various countries in Europe and the USA that is transmitted by ticks of the genus *Ixodes*. With the increasing incidence of HGA in the USA, especially in regions of the Midwest and Northeast, some experimental studies have shown that the agent of HGA may induce clinical disease in horses, besides protecting the subsequent infection, which is important when considering the possibility of

horses act as reservoir and source of infection for human granulocytic anaplasmosis<sup>(41)</sup>. Various mammals, such as horses, cattle, goats, dogs, and cats, have important roles in the propagation of *A. phagocytophilum* and its transmission to humans.

## Conclusion

The presence of anti-*A. phagocytophilum* antibodies in horses of the Mounted Police of Rio de Janeiro State, regardless of the place they are bred, shows that the parasite is enzootically present in horses of the urban region of Rio de Janeiro and that animal sex does not interfere in the susceptibility to the parasite.

## Acknowledgements

We would like to thank the Military Police of Rio de Janeiro state for making their animals available for the present study. Thanks also to the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) and to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

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