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Institutionen för kliniska vetenskaper

Seroprevalence of *Rickettsia rickettsii* and *Rickettsia amblyommii* in horses in three municipalities in the state of Pará, Brazil

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Seroprevalens av *Rickettsia rickettsii* och *Rickettsia amblyommii* hos hästar i tre kommuner i staten Pará, Brasilien

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ABSTRACT

Brazilian Spotted Fever (BSF) is caused by *Rickettsia rickettsii* and is considered to be the most important zoonotic tick-borne disease in Brazil. The disease seems to be reemerging and cases have been documented in several states, but very little is known about the occurrence in the Amazon basin. There are no cases of BSF reported in this area but medical care and diagnostic tools are not always available, therefore it is possible that cases are occurring without being diagnosed. Because of the severity of the BSF pathology, this disease must be considered to be of great public health importance.

Rickettsia rickettsii is the most pathogenic rickettsia species and because the agent more frequently occurs in horses than in humans, horses may be used as sentinel animals, even though there are no descriptions of clinical signs or symptoms in horses seropositive to *R. rickettsii*. Monitoring rickettsial seroprevalence in horses is therefore a valuable action for increasing the possibility to prevent Brazilian Spotted Fever in humans.

To provide epidemiological input on BSF, the seroprevalence of *Rickettsia rickettsii* and *Rickettsia amblyommii* was determined among horses in three municipalities (Óbidos, Brasil Novo, Santarém) in the state of Pará, northern Brazil, through serological testing by indirect immunofluorescence assay (IFA). Three groups of horses were included in the study, namely; farm horses, urban horses and sport horses. The results were used to evaluate whether there were differences between the three groups studied regarding the prevalence of seropositivity and to assess whether urban horses and horses used in sport are a risk of contamination for animals raised on farms, which represent the vast majority of the horse population in the state of Pará. Furthermore, the aim was also to detect possible associations between seroprevalence and determinants like gender, age, breed category and area.

By systematic cluster sampling, sera from a total of 436 horses were collected. Immunofluorescence assay was performed using vero cells infected with *R. rickettsii* (strain Tiaçu), and *R. amblyommii* (strain Ac37). Titers equal to and greater than 64 were considered positive. Sera from the positive samples were diluted in two-fold increments until reaching endpoint-titration.

Of the 436 sera tested, 37 (8.5%) reacted to *R. rickettsii* and 85 (19.5%) to *R. amblyommii*. The *R. rickettsii* titers ranged from 64 to 512 and the *R. amblyommii* titers ranged from 64 to 16384. In Brasil Novo the seroprevalence was significantly higher than in Santarém. The reason for this is not known. None of the determinants sex, age, breed category or area had any significant association with an increased seroprevalence. Nor did we see endpoint antibody titers that were higher than what can be expected in a non-endemic area.

SAMMANFATTNING

Brazilian Spotted Fever (BSF) orsakas av *Rickettsia rickettsii* och anses vara den allvarligaste zoonotiska fästingburna sjukdomen i Brasilien. Sjukdomen verkar återigen vara på uppgång och fall har dokumenterats i flera stater men ännu ej i Amazonas området. Det är dock möjligt att fall förekommer utan att de diagnostiseras pga. den bristande sjukvården i detta område. Med tanke på dess allvarliga karaktär måste BSF anses vara av stor vikt för folkhälsan.

Rickettsia rickettsii är den mest patogena rickettsia-arten och pga. att den i större utsträckning förekommer hos häst än hos människa, lämpar sig hästen som sentineldjur, trots att inga kliniska tecken har påvisats hos hästar seropositiva för *R. rickettsii*. Övervakning av seroprevalens av rickettsie hos hästar är därför en lämplig åtgärd för att öka chanserna att kunna förhindra BSF hos människor.

För att bidra med epidemiologisk kunskap om BSF, bestämdes seroprevalensen för *Rickettsia rickettsii* och *Rickettsia amblyommii* med hjälp av indirekt immunofluorescence (IFA), hos hästar i tre kommuner (Óbidos, Brasil Novo, Santarém) i staten Pará, norra Brasilien.

Tre grupper av hästar inkluderades i studien; gårdshästar, stadshästar och sporthästar. Resultaten från studien användes för att utvärdera huruvida skillnader fanns mellan de tre grupperna, med avseende på prevalens för seropositivitet och för att utvärdera huruvida stadshästar och sporthästar är en kontaminationsrisk för gårdshästar, vilket utgör den övervägande majoriteten av hästar i Pará. Syftet var även att detektera möjliga samband mellan seroprevalens och determinanter såsom kön, ålder, ras och kommun.

Sera från totalt 436 hästar samlades in genom systematisk klusterprovtagning. IFA utfördes med *R. rickettsii* (strain Taiacu) och *R. amblyommii* (strain Ac37) infekterade vero celler. Titrar motsvarande eller högre än 64 bedömdes positiva och spädningsserier gjordes därefter från de positiva proverna.

Av de 436 serumproverna som testades, reagerade 37 (8.5%) på *R. rickettsii* och 85 (19.5%) på *R. amblyommii*. Titrarna för *R. rickettsii* varierade från 64 till 512, och titrarna för *R. amblyommii* varierade från 64 till 16384. Seroprevalensen i Brasil Novo var signifikant högre än i Santarém men orsaken till detta är okänd. Ingen av de studerade determinanterna kön, ålder, ras eller kommun hade något signifikant samband med ökad seroprevalens. Vi såg inte heller någon antikroppstiter som var högre än vad som kan förväntas i ett icke-endemiskt område.

INTRODUCTION

Rickettsiae

Disease caused by organisms within the genus of rickettsiae can be divided into three biogroups, namely; the spotted fever group, the typhus group and the scrub typhus group. *R. rickettsii* and *R. amblyommii* are obligate intracellular bacteria belonging to the spotted fever group (SFG) of the genus *Rickettsia* within the family Rickettsiaceae in the order Rickettsiales. These organisms are non-motile, pleomorphic, Gram-negative and host cell dependent. Organisms belonging to the family Rickettsiaceae generally target endothelial cells, macrophages and leukocytes. The genus *rickettsia* has a predilection for small blood vessels, targeting the endothelial cells where they enter into and replicate in the cytoplasm, while inducing cytotoxic effects (Quinn, 2002).

Specific arthropods are needed for the transmission of most rickettsiae, therefore diseases caused by these organisms tend to occur in specific geographical regions (Quinn, 2002). Today the geographical distribution of *Rickettsia rickettsii* is restricted to North, Central and South America. Disease caused by *R. rickettsii* has gained many names over the years and the most common and generally used is Rocky Mountain Spotted Fever (RMSF), named after the region in North America where it was discovered. Brazilian Spotted Fever (BSF) is the name currently being used in Brazil (Labruna, 2009).

Vectors

Rickettsioses is most often transmitted by ticks, but can also be transmitted by fleas, lice and mites. Among the Spotted Fever Group Rickettsiae, ticks belonging to the family Ixodidae act as vectors, reservoirs and amplifiers (Parola, 2005). The tick, *Amblyomma cajennense*, especially in its immature stages, is considered to be the main vector of Brazilian Spotted Fever in Brazil. However, other ticks have also been suggested to act as potential vectors. Pinter and Labruna (2006) verified *Amblyomma aureolatum* as a vector in a study performed in the state of São Paulo and Cunha et al. (2009) recently detected *Rickettsia rickettsii* in *Rhipicephalus sanguineus*, in the state of Rio de Janeiro.

Through transovarial and transstadial transmission, *Amblyomma cajennense* makes it possible for *R. rickettsii* to maintain in the environment (Souza et al., 2008). Horses, capybaras (*Hydrochoerus hydrochaeris*) and tapirs (*Tapirus terrestris*) are considered to be primary hosts for all parasitic stages of *A. cajennense*, but the nymphs and larvae of *A. cajennense* have low host specificity and can therefore be aggressive to humans and dogs as well (Lemos et al., 1996). In a study performed by Horta (2009), the results indicated that opossums under laboratory conditions, can also act as amplifier hosts for *A. cajennense* larvae and nymphs.

The presence of *A. cajennense* and the infestation levels on horses have been shown to be strongly affected by vegetation conditions of the pastures. A strong statistical association was found between the presence of mixed overgrowth pastures and high infestation on the horses in the state of São Paulo, Brazil (Labruna, 2001). Infestation in secondary hosts, such as dogs

and humans, is more likely to occur when there is a high infestation by *A. cajennense* among horses (Lemos, 1996; Labruna et al., 2002). An efficient method to reduce human infection is therefore to control the *A. cajennense* infestation on horses, especially in Brazilian Spotted Fever endemic areas. However, several studies have shown that *R. rickettsii* infections are very low among *A. cajennense* populations in endemic areas, where around 1% or less of the ticks are found infected by the bacterium (Guedes et al. 2005; Sangioni et al. 2005). This is believed to be the effect of the pathogenesis of *R. rickettsii* to the tick. High mortality has been seen in ticks infected with *R. rickettsii* and infected ticks produce fewer progeny than uninfected ticks. The mechanism for rickettsia-induced mortality is still unclear but it implies the need of carriers for horizontal transmission, in order to create new lines of infected ticks and to be able to maintain in nature (Niebylski et al., 1999).

Brazilian Spotted Fever

Since BSF is dependent on ticks for transmission to human hosts and *Amblyomma cajennense* is only an occasional ectoparasite of humans, the disease is generally focal and sporadic and do not tend to come in strong epidemics or surges (Silva, 2004). Nevertheless, many experts claim Brazilian Spotted Fever to be the most important tick-borne disease in Brazil (Lemos, 2001; Horta, 2004; Sangioni, 2005). BSF is acquired through the bite of an infected tick that stays attached to the host for at least four to six hours. Within the human host, the pathogenic rickettsiae localize and multiply in endothelial cells, resulting in vasculitis. The onset of the disease is often characterized with high fever and headache, which may be accompanied by nonspecific symptoms such as nausea, vomiting, diarrhea, anorexia and abdominal pain. With this clinical picture, misdiagnosing is not unusual, especially since the typical rash of BSF, at its earliest, occurs on day three of fever (Raoult, 1997).

BSF was first reported in Brazil in 1929. Between the 1940s and the 1980s the number of reported cases of BSF dropped markedly. Since then, the disease seems to be reemerging and cases have been documented in several states, particularly in the southeast region of the country where it is considered to be endemic (Lemos, 2001; Galvao, 2003). Very little is known about the occurrence of BSF in the Amazon basin but the area is considered to be non-endemic. It has been shown that in the state of Rondonia, Western Amazon, a number of human patients from rural areas were presented with fever of unknown cause and also claiming to have had a recent tick bites. It cannot be excluded that tick-borne Rickettsia might be a possible infectious agent in these cases (Labruna, 2004).

Even though many *Rickettsia spp.* have the potential to be pathogenic to humans, certain components minimize the risk of exposure. The tick, for example, must have a tendency to bite humans and the bacterium has to be transmitted through the tick bite, which demands that the bacterium can localize in the salivary glands of the tick (Niebylski, 1997). Other elements that affect the frequency of tick-borne rickettsioses are the amount of tick vectors, the prevalence of infection within ticks and the amount of natural hosts that come in contact with humans (Parola, 2001).

People with antibody reactivity against *R. rickettsii* can be asymptomatic, but the disease has always been associated with high lethality. However, with early specific anti-rickettsial therapy, this high lethality can be reduced (Lemos, 2001). At a conference in 2009, Labruna presented data of 128 confirmed cases of BSF in the state of São Paulo from 2005 to 2007, with a case fatality risk of 29%. In addition to *R. rickettsii*, several other tick-borne species of *Rickettsia* have been shown to cause human infection, e.g. *R. felis*, *R. parkeri*, and *R. massiliae*.

***Rickettsia rickettsii* in horses**

In a study by Batista et al. (2010), six (8.45%) horses from a non-endemic area were seropositive against *R. rickettsii*, with titers $\leq 1:1024$. Tamekuni (2010) studied another non-endemic area and found that fifteen (5.5%) horses were seropositive against *R. rickettsii* with titers $\leq 1:512$. According to Lemos et al., (1996) there are no descriptions of clinical signs or symptoms in horses, even when they have high titers ($\geq 1:1024$) against *R. rickettsia*. In his study, titers $\geq 1:1024$ against *R. rickettsia* in horses were associated with confirmed human cases of BSF in the same area. His results indicated that the seroprevalence was significantly higher in the endemic area than in the control population. This suggests that it might be possible to use horses as sentinel hosts for human infection in endemic areas of BSF. Several studies have shown similar patterns in BSF endemic areas, namely: a high frequency of serologically positive horses followed by a lower frequency in dogs, and an even lower frequency or absence of serologically positive humans (Horta, 2004; Lemos, 1994; Lemos et al., 1996). The fact that horses, and not dogs or humans, are primary hosts of *A. cajennense*, can explain why horses have a higher infection incidence of *R. rickettsii* (Horta, 2004). Therefore Sangioni (2005) recommend surveys of horse sera as a method for BSF surveillance in areas where humans are exposed to *A. cajennense* ticks. This way, potentially, endemic areas could be identified before human cases occur. In the state of Pará, the seroprevalence of *Rickettsia* in horses has previously not been studied but the region is considered to be non-endemic (A. H. Hamad Minervino, 2012, personal communication).

***Rickettsia amblyommii* in horses**

When *Rickettsia amblyommii* first was isolated from the tick *Amblyomma americanum*, it was shown to be nonpathogenic to laboratory animals. This finding, plus the fact that humans were frequently exposed to *Amblyomma americanum* without apparent illness, suggested that *R. amblyommii* also was nonpathogenic to humans (Burgdorfer et al. 1981). However, Apperson (2008) recently performed a study in North Carolina, USA, where he proposed that some cases reported to have been caused by *R. rickettsii*, in fact may have been caused by *R. amblyommii* transmitted from *A. americanum*, due to the fact that the diagnose not always had been confirmed. Many times they have instead been classified as probable cases, based on symptoms, serology, exposure to ticks etc.

In 2004, Labruna detected *R. amblyommii* in *Amblyomma cajennense* and *Amblyomma coelebs* ticks collected from the western Amazon forest of Brazil. By doing so it was the first

evidence of *R. amblyommii* in ticks other than *A. americanum* and the first time it was detected outside the United States. In that study, DNA sequences of PCR products from *A. cajennense* and *A. coelebs* were found to be 100% (350/350) identical to *Rickettsia amblyommii*. No similar study have been made in the state of Pará but with such high prevalence in a region close by, it is not unlikely to find high prevalence of *R. amblyommii* in the state of Pará as well, both in ticks and horses (Labruna, 2012, personal communication).

Horses in the state of Pará, Brazil

Farm horses

Only in the state of Pará the equine population is larger than 280 000 animals, playing a major socio-economic roll. A major part of the horses are found at farms where they are used for routine services of the farm such as cattle management and for transportation in rural communities. (Hamad Minervino, 2012, personal communication).

Urban horses

Urban horses, sometimes referred to as cart horses, are extensively used in urban and urban fringe areas in Brazil. They often walk long distances, up to 40 km each day, performing hard labor (Freitas, 2010). These animals are mainly used by poor people as means of transportation or for collecting recycling materials. The horses usually lack the possibility of veterinary care and are often in poor nutritional status. They are of great importance in daily life and sudden poor health can further reduce a potentially already low income (Hamad Minervino, 2012, personal communication).

Sport horses

These horses are of high economic value, are well nourished and well taken care of. As they participate in competitions, they travel to a great extent between cities and are kept in a way that make them potential sources of spreading infectious diseases (Hamad Minervino, 2012, personal communication).

OBJECTIVE

Aims

The aims of this study were to determine the seroprevalence of *Rickettsia rickettsii* and *Rickettsia amblyommii* among horses in three municipalities in the state of Pará, northern Brazil, and to identify associations with horse characteristics such as sex, age, breed category and type of horse.

MATERIAL AND METHODS

Study design

This study was part of a more extensive study which includes all the 26 biggest municipalities in the state of Pará, Brazil. That study is currently being performed by prof. Antonio Humberto Hamad Minervino, Federal University of Western Pará (UFOPA). In this single point study, three of those municipalities were included, namely Santarém (2°25'48"S 54°43'12"W), Óbidos (1°54'0" S, 55°31'0"W) and Brasil Novo (3°15'42.98"S, 52°40'4.08"W), state of Pará, Northern Brazil. A systematic cluster sampling was performed in all three municipalities. The information about the number of horses and farms in each municipality was provided from the Brazilian institute of geography and statistics, Municipal Agricultural Production, and is shown in table 1. Owners of the selected farms were not informed prior to the visit. If the owners did not have any horses or if they did not agree to participate in the study, the closest neighboring farm was selected instead.

Table 1. Number of farms included in the study and total number of farms and horses in Óbidos, Brasil Novo and Santarém, state of Pará, Brazil

City name	No. of farms with horses	Total no. of horses	Average farm size (horses)	No. of farms sampled
Óbidos	980	4975	5.1	9
Brasil Novo	1135	3751	3.3	11
Santarém	648	2836	4.4	6

At each farm visit, blood samples from all horses present were collected for analyses and information about determinants such as sex, age, breed category and area were gathered.

Collection of samples

Between August 2011 and October 2012, sera from a total of 436 horses were collected.

Serum tubes were used for blood sampling, which was performed aseptically via the jugular vein. Blood samples were then transported to the laboratory in a cooling bag with ice and centrifuged ($1,500 \times g$ for 15 minutes). The samples were then obtained and aliquoted into Eppendorf tubes, labeled and kept at -20°C until analysis.

Laboratory analysis

Rickettsia diagnosis can be performed through serological techniques, isolation, genome and antigen detection. The most current method used for this diagnosis is serology using indirect immunofluorescence assay (IFA) (Lascola; Raoult, 1997; Horta et al., 2004). In order to detect a SFG infection, assays with a single *Rickettsia* antigen (e.g., *R. rickettsia*) can be used. This is possible because all SFG *Rickettsia* share common outer membrane antigens, which results in cross-reactions between *Rickettsia* species (Lascola; Raoult, 1997). However, the cross-reactivity makes it difficult to determine which *Rickettsia* species is responsible for infection. In order to do so, it is recommended to test serum against all known Rickettsia species, relevant to the area studied. By comparing titers among different antigens, a probable etiologic agent can be determined (Horta et al., 2004; Pacheco et al., 2007).

All laboratory analyses were carried out at the University of São Paulo (USP), according to the protocol by Horta et al. (2004). Indirect immunofluorescence assay was performed through serum samples reaction to Vero cells infected with *R. rickettsii* (strain Taiacu), and *R. amblyommii* (strain Ac37) fixed on microscope slides for immunofluorescence. Rickettsia species were cultivated in Vero cells until nearly 100% of the cells were infected, at which time they were harvested. These cells were then left to air dry on multiwell Teflon-coated glass slides and fixed in acetone. The slides were then stored at -20°C until used. These slides were prepared and provided by the Faculty of Veterinary Medicine, University of São Paulo.

Initial analyses (trials) were performed on all the collected samples in order to determine which samples were positive. Before the horse serum was added to the frozen slides, the slides were left in PBS¹-bath for 10 minutes at room temperature, thereafter leaving them to dry at room temperature. Samples, positive and negative controls were diluted to 1:64 with PBS in ELISA-type microplates. To accomplish this dilution, 3 µl sera was added to 189 µl PBS. 20 µl of each diluted sample, positive and negative control were then transmitted to wells on the slides. See figure 1.

Figure 1. Example of a slide during trials

	Positive control	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
Trials Rickettsia Slide 1	1:64	1:64	1:64	1:64	1:64	1:64
	1:64	1:64	1:64	1:64	1:64	1:64
	Negative control	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10

¹ PBS (phosphate buffer pH 7.2) containing NaCl- 0.15M, Na₂HPO₄ PA-0,0072 M, NaH₂PO₄ PA- 0,0028 M and distilled water.

The slides were placed upon wet surface and incubated at 37°C for 30 minutes in a humid chamber. The slides were then rinsed once with PBS and washed twice for 10 minutes per wash in PBS, leaving them to dry in room temperature. When dried, 20 µl of diluted conjugate (anti-horse Ig, 1:1200) were added to each well on the slides and then incubated for 30 min. Finally, the slides were washed twice for 10 minutes per wash with washing buffer plus Evans Blue (0.1 %) at a ratio of 0.3 ml of Evans Blue per 100 ml washing buffer. When kept to dry in room temperature, the slides were kept away from light sources.

For the reading of the slides, 2-3 drops of buffered glycerin (pH 9.0 ±0.5) were added on each slide, covering it with a coverslip. While reading, slides were kept in the dark. In each slide, negative and positive controls were tested. When serum showed to be reactive at 1:64 dilutions, it was considered to contain antibodies against rickettsiae.

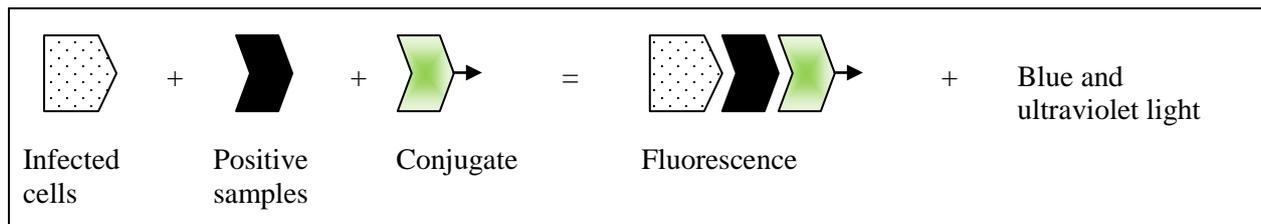
Next, the sera from the positive samples were diluted in two-fold increments with PBS, starting from a 1:64 dilution, continuing until reaching endpoint-titration (1:128, 1: 256, 1:518, 1:1024 etc.). Preparation of the slides were done as during the trials except that sera from only two individuals were added to each slide, as shown in figure 2.

Figure 2. Example of a slide during titration

	Positiv control	Sample 1				
Titration Rickettsia Slide 1	1:64	1:64	1:128	1:256	1:518	1:1024
	1:64	1:64	1:128	1:256	1:518	1:1024
	Negative control	Sample 2				

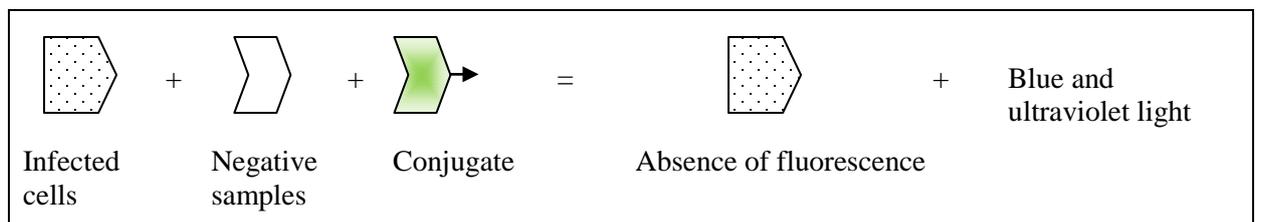
An immunofluorescence microscope with a 40X lens was used for the reading and interpretation of the reactions. When presence of intracellular fluorescence compatible with the standard format of rickettsiae, within the vast majority of cells (90-100 %) and in the extracellular space, the interpretation is that the sample is reactive, as in the positive control. The principle of reaction is shown below in figure 3.

Figure 3. Reaction.



When no reaction is seen, as in the negative control, there is absence of intracellular fluorescence compatible with the standard format of rickettsia. The principle of no reaction is shown below in figure 4.

Figure 4. No reaction



If a sample of serum or plasma proved reactive up to dilutions of 1:1024, continued testing of this sample was performed in a new slide. When sera was shown to be reactive to both *R. rickettsii* and *R. amblyommii* a titer at least four-fold higher than that observed for the other *Rickettsia* species tested, were considered homologous to the infecting species (Horta, 2004). Sera that were not homologous were classified as “undecided”.

Associations between determinants (sex, age, use, breed category and area) and the seroprevalence of *R. rickettsii* were tested using Chi-square test. This was done twice, once when excluding “undecided” samples and once when including them as “positive” when including them in order to increase the sample size. The age determinant were divided into four groups; < 1 year, 1-5 years, 6-10 years and ≥ 11 years. The breed determinant were divided into five groups; SRD (no defined breed), QM (Quarter Horse and mixes of Quarter Horse), Mules, Donkeys and Other (unknown breeds and breeds of Mangalarga/Paulista, Painthorse and Mestica).

RESULTS

Distribution of antibody titers

As seen in table 2, the highest antibody endpoint titer for *R. rickettsii* was 1:512 and this was seen in all three municipalities studied. The highest antibody endpoint titer for *R. amblyommii* was also 1:512 in both Óbidos and Santarém but in Brasil Novo 1:16384.

Table 2. Endpoint antibody titers by indirect immunofluorescence assay (IFA) for *Rickettsia rickettsii* and *Rickettsia amblyommii* antigen in horses from the municipalities of Óbidos, Brasil Novo and Santarém, state of Pará, Brazil

Municipality	Endpoint antibody titer								
	64	128	256	512	1,024	2,048	4,096	8,192	16384
Óbidos									
(n=106)									
<i>R. rickettsii</i>	4	1	2	1					
(%)	(3.8)	(0.9)	(1.9)	(0.9)					
<i>R. amblyommii</i>	5	5		1					
(%)	(4.7)	(4.7)		(0.9)					
Brasil Novo									
(n=162)									
<i>R. rickettsia</i>	8	10	10	4					
(%)	(4.9)	(6.2)	(6.2)	(2.5)					
<i>R. amblyommii</i>	1	7	10	20	15	5	6	4	4
(%)	(0.6)	(4.3)	(6.2)	(12.3)	(9.3)	(3.1)	(3.7)	(2.5)	(2.5)
Santarém									
(n=168)									
<i>R. rickettsia</i>	4	3	2	1					
(%)	(2.4)	(1.8)	(1.2)	(0.6)					
<i>R. amblyommii</i>	6	2	1	1					
(%)	(3.6)	(1.2)	(0.6)	(0.6)					

Overall prevalence of *R. rickettsii* and *R. amblyommii*

As seen in table 3, the overall seroprevalence of *R. rickettsia* in horses was 8.5% (37/436). The overall seroprevalence of *R. amblyommii* was 19.5% (85/436), while 2.3% (10/436) of the samples showed a positive reaction to the spotted fever group but could not be determined more closely.

Table 3. Results of indirect immunofluorescence assay (IFA) for antibodies to *Rickettsia rickettsii* and *Rickettsia amblyommii* in horses from the municipalities of Óbidos, Brasil Novo and Santarém, state of Pará, Brazil

Municipality	Type of horses							
	Urban horses		Farm horses		Sports horses		Total	
n*	IFA reactive sera † (%)	n*	IFA reactive sera † (%)	n*	IFA reactive sera † (%)	n*	IFA reactive sera † (%)	
Óbidos	3		95		8		106	
<i>R. rickettsii</i>	1 (33.3)		6 (6.3)		0 (0)		7 (6.6)	
<i>R. amblyommii</i>	0 (0)		10 (10.5)		0 (0)		10 (10.6)	
<i>Undetermined</i>	0 (0)		1 (01.1)		0 (0)		1 (0.9)	
Brasil Novo	6		143		13		162	
<i>R. rickettsii</i>	0 (0)		22 (15.4)		0 (0)		22 (13.6)	
<i>R. amblyommii</i>	2 (33.3)		57 (39.9)		8 (61.5)		67 (41.4)	
<i>Undetermined</i>	0 (0)		7 (4.9)		0 (0)		7 (4.3)	
Santarém	81		68		19		168	
<i>R. rickettsii</i>	6 (7.4)		2 (2.9)		0 (0)		8 (4.8)	
<i>R. amblyommii</i>	2 (2.5)		4 (5.9)		2 (10.5)		8 (4.8)	
<i>Undetermined</i>	1 (1.2)		0 (0)		1 (5.3)		2 (1.2)	
Total	90		306		40		436	
<i>R. rickettsii</i>	7 (7.8)		30 (9.8)		0 (0.0)			
<i>R. Amblyommii</i>	4 (4.4)		71 (23.2)		10 (25)			
<i>Undetermined</i>	1 (1.1)		8 (2.6)		1 (2.5)			

* Total number of sera tested.

† Number of positive sera showing titers ≥ 64 for *R. rickettsii*/ *R. amblyommii* antigen.

Prevalence on the basis of determinants

As seen in table 4, in total, 40 sport horses were included in this study, none of them were solely positive as *R. rickettsii*, whereas 25% (10/40) of them were positive to *R. amblyommii*. There were no statistical difference between urban horses and farm horses. None of the determinants; sex, age or breed category, had any significant association to an increased seroprevalence of *R. rickettsii* in horses. In Brasil Novo, the seroprevalence of *R. rickettsii* were significantly higher than in Santarém.

Table 4. Prevalence of *Rickettsia rickettsii* positive samples according to the determinants: gender, breed, age, use and area

Determinants	<i>R. rickettsii</i> when including undetermined samples		<i>R. rickettsii</i> when excluding undetermined samples	
	No.	(%)	No.	(%)
Gender				
Male	21/257	8.2 ^a	17/257	6.6 ^a
Female	11/166	6.6 ^a	6/166	3.6 ^a
Breed [◊]				
SRD	25/262	9.5	18/262	6.9
QM	3/111	2.7	1/111	0.9
Mule	1/6	16.7	1/6	16.7
Donkey	1/6	16.7	1/6	16.7
Other	1/48	2.1	1/48	2.1
Age [♦]				
< 1 year	0/15	0.0 [*]	0/15	0.0 [*]
1-5 year	6/108	5.6 ^a	5/108	4.6 ^a
6-10 year	8/126	6.3 ^a	8/128	6.3 ^a
≥ 11 year	5/33	15.2 ^a	4/33	12.1 ^a
Use				
Urban	8/87	9.2 ^a	7/87	8.0 ^a
Farm	38/306	12.4 ^a	30/306	9.8 ^a
Sport	1/40	2.5 [*]	0/40	0.0 [*]
Area				
Óbidos	8/106	7.5 ^{ab}	7/106	6.6 ^{ab}
Brasil Novo	29/162	17.9 ^b	22/162	13.6 ^b
Santarém	10/168	6.0 ^a	8/168	4.8 ^a

* At least 20% of expected frequencies are less than 5, chi-square test not performed.

[◊] SRD: no defined breed. QM: Quarter Horse and mixes of Quarter Horse. Other: unknown breeds and breeds of Mangalarga/Paulista, Painthorse and Mestica. Only the SRD group fulfilled the requirements of chi-square test, therefore comparison between groups has not been made.

[♦]Four horses with unknown age were not included in this table.

^{ab}Values in the same column, within determinant, with the same superscript are not statistically different (p>0.05)

DISCUSSION

The present study showed that the highest antibody endpoint titer for *R. rickettsii* was 1:512 and this was seen in all three municipalities studied. Since the state of Pará is considered to be non-endemic and this study shows low endpoint titers, this corresponds well to the study of Lemos et al. (1996), that horses from endemic areas have a higher antibody titer than in non-endemic areas. In a study by Horta et al. (2004), antibody titers against *R. rickettsii* in BSF-endemic farms were $\geq 1:1024$. Sangioni et al. (2005) performed a study in the same area but from farms considered non-endemic (no confirmed human cases of BSF) and no samples from horses or humans showed positive reactions against *R. rickettsii* antigens. To rule out false high *R. rickettsii* seroprevalence due to cross-reactions to other rickettsia species, *Rickettsia amblyommii* was also studied since it has been shown to have high seroprevalence in the *A. cajennense* tick in an adjacent state to Pará (Labruna, 2012, personal communication). The highest antibody endpoint titer for *R. amblyommii* was also 1:512 in both Óbidos and Santarém, and was 1:16384 in Brasil Novo.

In using serological diagnostic methods, there have been difficulties determining whether the antibodies have been stimulated by *R. rickettsii*, *R. amblyommii*, another SFG rickettsia, or even a crossreaction from a non-rickettsia antigen. Therefore we can only conclude that the agent, or an organism that is serologically related, is present in the area studied, although at low frequencies. We can suspect that BSF is present in the area but cannot solely be proven on the basis of serological evidence. The bacterium has still not been isolated from ticks nor humans in this area. However, in this poorly developed part of northern Brazil and in the isolated parts of Amazonas, medical care and diagnostic tools are not always available, therefore it is possible that cases are occurring without being diagnosed (Hamad Minervino 2012, Personal communication). According to Sangioni (2005) most horses and dogs from BSF-endemic areas are shown to have positive serological reactions against *R. rickettsii* antigens, whereas no horses or dogs have positive reactions in non-endemic areas, even though they have been exposed to the vector. According to this, the state of Pará should not have had any positive reactions, since it is considered to be non-endemic.

As described in the background, prevalence of *R. rickettsii* can be minimized or even inhibited when a second less pathogenic *Rickettsia* species infect the same tick population. It is possible that the higher seroprevalence of *R. amblyommii* within areas studied minimizes the spreading of *R. rickettsii*, therefore making the state of Pará non-endemic. However, Brasil Novo had the highest prevalence of both *R. amblyommii* and *R. rickettsia*, which do not strengthen the hypothesis previously described.

We found that Brasil Novo had a significantly higher seroprevalence of *R. Rickettsii* than Santarém, and numerically higher than Obidos. A possible explanation for this could be differences in farm management techniques, such as grazing pressure and rotational grazing schemes, if pasture consists of naturally forested habitats or open grazing areas, the amount of wild animals in the area working as tick carriers, or how often and what time of year owners treat their animals against ticks. Definitive conclusions on this matter require a more

extensive study regarding determinants in these specific areas. Because the agents studied are endemic in the southeast region of the country, it is also possible that they may have spread from this region, reaching Brazil Novo before Santarém and Obidos.

There were no differences according to gender and there were too few horses of some breed groups to be able to test for possible differences. Also, there were no statistically significant differences according to age, but a numerical increase in the prevalence with age. This result would be expected, because older horses would have been more exposed to ticks during a longer period of time. Higher antibody positivity amongst the older horses would then suggest that the area have been exposed to the agents studied during quite some time. In order to gain a larger sample size, samples classified as “undecided” were categorized into the group of samples that were positive to *R. rickettsii*. However, this did not change any results.

Except for the mere epidemiological purpose, one aim of this study was also to assess whether urban horses and horses used in sport are a risk of contamination for animals raised on farms, which represent the vast majority of the horse population in the state of Pará. There were no statistical difference between urban horses and farm horses. Unfortunately, few sport horses were available for sampling therefore making it difficult to find statistical associations. However, there was a strong indication that the prevalence in sport horses were lower than for urban and farm horses because none of the 40 sport horses were positive for *R. rickettsia*. The reason for this is not known, but one explanation could be that these horses are of such a high value that they get treated against ticks more frequently than both urban and farm horses. They might also be held more restricted, on better pastures, spending more time in stables etc. According to this study, sport horses may not have a big role in the spreading of *R. rickettsii*, although an infected sport horse, traveling a lot, would have a great impact on spreading *R. rickettsii* to new areas.

In a study performed by Horta (2004), he discussed a possible inability of *R. rickettsii* to infect donkeys and that donkeys might have a higher resistance against infestation by *A. cajennense*. The results from this study showed that both donkeys and mules are able to be infected by *R. rickettsii*. Six mules and six donkeys were tested; one mule with a titer of 1:64 and one donkey with a titer of 1:64 were detected. A larger sample of mules and donkeys is necessary to be able to determine if there is a significant difference in prevalence between mules, donkeys and horses.

Because serology is a very subjective diagnostic method, it would have been of great value if all samples were analyzed by the same person, even if this increases the risk of systemic errors with readings consistently above or below the true value. In this study, 90% of the samples were analyzed by the same person, which gives a fairly high precision in the result.

Even though Pará is considered to be non-endemic, our study shows that there are seropositive horses present in the state, meaning there is also a risk that the disease can spread. Therefore, now is an appropriate time to perform ecological studies based on reliable data concerning even more determinants, in order to prevent the spreading of disease before

more areas become endemic. Because of the ticks' capacity of transmitting the disease and due to their high resistance to the environment in which they live, control of the tick population and spreading of the disease will be very difficult. However, because Labruna (2001) found a strong association between the presences of mixed overgrowth pastures and high infestation on the horses, control of pastures could be a way for non-endemic areas to remain free. Also by continuously removing and treating against ticks, humans will be less likely to come in contact with infectious vectors. It is also of great importance that increased public and physician education takes place regarding BSF, in order to recognize the disease in early stages.

Conclusion

Horses' antibody positive to *R.rickettsii* and *R. amblyommii* were found in all three municipalities studied. However, endpoint antibody titers for *R. rickettsia*, $\leq 1:512$, were not higher than expected in a non-endemic area. We found that Brazil Novo had a significantly higher seroprevalence of *R. rickettsii* than Santarém. There were no associations between the determinants; gender, age or breed category, of the horses and the seroprevalence of *R. rickettsii*. Nor did we see any significant difference between urban horses and farm horses.

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