

PREVALENCE OF ANTIBODIES TO SPOTTED FEVER GROUP RICKETTSIAE IN HUMANS AND DOMESTIC ANIMALS IN A BRAZILIAN SPOTTED FEVER–ENDEMIC AREA IN THE STATE OF SÃO PAULO, BRAZIL: SEROLOGIC EVIDENCE FOR INFECTION BY *RICKETTSIA RICKETTSII* AND ANOTHER SPOTTED FEVER GROUP RICKETTSIA

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Abstract. In serum samples obtained from all the healthy humans, horses, dogs, and donkeys present on three farms in the Pedreira Municipality, an endemic area for Brazilian spotted fever, an indirect immunofluorescence assay (IFA) detected antibodies against *Rickettsia rickettsii* in 17 (77.3%) horses, 5 (31.3%) dogs (titers ranging from 64 to 4,048), and none of 4 donkeys or 50 humans. Five canine and eight equine sera with high antibody titers to *R. rickettsii* were also tested by IFA against *R. bellii*, *R. akari*, and *R. africae* antigens. Sera from two horses and two dogs that showed similar high antibody titers against two rickettsial antigens were evaluated after cross-absorption. Sera from seven horses and two dogs contained antibodies specific for *R. rickettsii*, and one dog serum had antibodies against a *Rickettsia* species very closely related to *R. africae*. The latter may have been caused by infection with the recently identified COOPERI strain.

INTRODUCTION

The genus *Rickettsia* comprises obligate intracellular bacteria, many of which cause zoonotic diseases in different parts of the world, whereas other *Rickettsia* species are considered non-pathogenic or of unknown pathogenicity for humans.¹ Recent studies on genotypic characterization of rickettsiae have divided them into three groups: the ancestral group (AG), composed of *Rickettsia bellii* and *R. canadensis*, associated with ticks; the typhus group (TG), composed of *R. prowazekii* and *R. typhi*, associated with lice and fleas; and the spotted fever group (SFG), which includes more than 20 valid species associated with ticks, one species (*R. akari*) associated with mites and one (*R. felis*) with fleas.^{1–3}

The indirect immunofluorescence assay (IFA) is currently the test of choice for serologic diagnosis of rickettsial infection in humans and animals.^{1,4} However, cross-reactive antibodies between *Rickettsia* species are often observed, rendering difficult the serologic identification of the *Rickettsia* species involved in an infection. The geographic origin of the infection has been one of the best indicators of species identity. Testing a clinical serum against the possible *Rickettsia* species known to occur in a given area is ideal because often homologous antibody titers are higher than heterologous antibody titers. In some cases, the differences in titers may be great enough to differentiate among the rickettsial species potentially stimulating the immune response.⁴ On the other hand, especially among more closely related rickettsial species, a heterologous antibody titer may be as high as the homologous antibody titer. In this situation, the use of other techniques such as cross-absorption may enable the differentiation between homologous and heterologous antibodies.⁴

Rickettsia rickettsii, an SFG species, is the etiologic agent of Brazilian spotted fever (BSF), a currently nationally notifiable disease in Brazil. Lethal cases of BSF among humans have been reported in the state of São Paulo for more than 70 years.⁵ For many decades, *R. rickettsii* was the only *Rickettsia* species known to infect ticks in Brazil. Recent studies have

isolated *R. bellii* and *R. amblyommii* from *Amblyomma* spp. ticks from Brazil, and there is molecular evidence for at least three distinct SFG rickettsial species in Brazil (Labruna MB, Walker DH, unpublished data).^{6–8}

The main vector of *R. rickettsii* in Brazil is the *Amblyomma cajennense* tick, which is also the main tick species infesting humans in the central and southeastern states of Brazil. Although horses, tapirs (*Tapirus terrestris*), and capybaras (*Hydrochaeris hydrochaeris*) are the primary hosts for *A. cajennense*, this tick may feed on any medium or large sized host when the tick population is large.⁹ These hosts include the domestic dog, which is often infested by *A. cajennense* in rural areas of Brazil.¹⁰

Serologic evidence for infection by SFG rickettsiae in humans and domestic animals from BSF-endemic areas has been demonstrated previously using only *R. rickettsii* antigens.^{11–15} In the present study, we evaluated infection by *Rickettsia* spp in horses, dogs, and humans from an area endemic for BSF by serologic methods using antigens from up to four *Rickettsia* species. In addition, some of the reactive sera were evaluated by cross-absorption testing.

MATERIALS AND METHODS

Study location. This study was conducted in three areas of Pedreira Municipality, State of São Paulo, where recent cases of BSF have been reported in humans.^{11,16} Two of these areas were farm 1 (22°44'19"S, 46°55'27"W) and farm 2 (22°47'03"S, 46°54'10"W), both located along the banks of the Jaguari River, and the third area was farm 3 (22°41'14"S, 46°53'17"W), which was located along the banks of the Camanducaia River. Human occupations were divided basically between livestock activity for men and household activities for women and children. Nevertheless, children did spend substantial time in outdoor activities. The three farms had similar ecologic features, including the presence of horses grazing on mixed overgrowth pastures, interposed with remote forest areas, which were in-

habited by large populations of free-living capybaras. In addition, all three farms had free roaming dogs with free access to pasture and forest areas. Previous studies of ticks collected on the pastures and from horses and dogs of these three farms allowed the identification of two tick species: *A. cajennense* and *A. cooperi*, the former occurring in very large populations.

Sera collection. During January and February 2001, blood samples were collected from 16 dogs (18 months to 7 years old), 22 horses (12 months to 25 years old), 4 donkeys (4 to 7 years old) and 50 humans (age ranging from 2 to 69 years). This sample represented approximately 90% of the human resident population and 100% of the domestic animals of the three farms. Blood samples were transported to the laboratory at room temperature where samples were centrifuged ($1,500 \times g$ for 10 minutes), and the sera were aliquoted into labeled microtubes and stored at -20°C until tested.

Indirect immunofluorescence assay. The IFA procedure followed the procedure described by Zavala-Velazquez and others.¹⁷ *Rickettsia rickettsii* (Sheila Smith strain) were cultivated in Vero cells and harvested when nearly 100% of the cells were infected. The infected cells were centrifuged at $12,000 \times g$ for 10 minutes, and the pellet was washed in 0.1 M phosphate-buffered saline (PBS), pH 7.4, centrifuged again, and resuspended in PBS containing 1% bovine calf serum and 0.01% sodium azide. Ten microliters of rickettsiae-infected cells were applied onto each of 12 wells on microscopic slides. The antigens on the slides were air-dried and then fixed in acetone for 10 minutes. Slides were stored at -20°C until used. Human and animal sera were diluted in two-fold increments with PBS starting from a 1:64 dilution. Ten microliters of diluted sera were added to each well of the antigen slides. The slides were incubated at 37°C for 30 minutes in a humid chamber. The slides were rinsed once, then washed twice for 10 minutes per wash in PBS. The slides were incubated with fluorescein isothiocyanate-labeled goat anti-human IgG, goat anti-horse IgG, or goat anti-dog IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) and washed as described earlier. The slides were mounted with Gel Mount (Biomed, Foster City, CA) under coverslips. The slides were read using an ultraviolet microscope (BX60; Olympus, Tokyo, Japan) at $400\times$ magnification. Serum was considered to contain antibodies against the rickettsiae if it displayed a reaction at the 1:64 dilution. In each slide, a serum previously shown to be non-reactive (negative control) and a known reactive serum (positive control) were tested.

All dog sera that reacted positively against the *R. rickettsii* antigen and eight horse sera showing high titers against *R. rickettsii* were also tested by IFA with *R. bellii* (strain Ac25), *R. akari* (strain MK), and *R. africae* (ESF5 strain) antigens. These three additional antigens were selected due to the following rationale: *R. bellii* has been recently isolated from *A. cooperi* ticks from the present study area;⁶ *R. akari* is antigenically closely related to *R. felis*, which has been detected in fleas collected from dogs from the studied area (Horta MC, Walker DH, unpublished data); and *R. africae* is genotypically closely related to strain COOPERI, which has been identified in *A. cooperi* ticks from the studied area.⁶ We used *R. africae* antigen because the isolate designated as strain COOPERI, once isolated in cell culture, was lost during passage in the laboratory and it is most closely related phyloge-

netically to *R. africae*.⁶ Sera showing, for a particular *Rickettsia* species, a titer at least four-fold higher than that observed for any other *Rickettsia* species tested were considered homologous to the *Rickettsia* species with the highest titer. However, if a serum showed the highest titer to *R. africae* (at least four-fold higher than titers to the other *Rickettsia* species), this antibody was considered to be elicited by the strain COOPERI, since *R. africae* and its natural vectors have never been described in the studied area or in South America. Similarly, if a serum showed the highest titer to *R. akari* (at least four-fold higher than titers to the other *Rickettsia* species), this antibody was considered likely to have been elicited by *R. felis*, since *R. akari* and its natural vectors have never been reported in the studied area.

Two IFA-positive horse sera that showed high titers against both *R. rickettsii* and *R. africae* (titers differing by at most a two-fold dilution), one IFA-positive dog serum that showed the same titer against *R. rickettsii* and *R. africae*, and one IFA-positive dog serum that showed the same titer against *R. rickettsii* and *R. akari* were selected for the cross-absorption study.

Serum cross-absorption. This procedure was performed with slight modifications of protocols previously described.^{18,19} Each rickettsial antigen was produced from renografin-purified rickettsiae from 30–40 150-cm^2 flasks of 100% infected Vero cells. The protein concentration of the antigen was determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL) on rickettsiae dissolved in 100 mM of Tris, pH 7.4, and 2% sodium dodecyl sulfate. After centrifugation of 0.250 mL of purified rickettsiae, a pellet containing approximately 1.5 mg of antigen was resuspended in 1 mL of the test serum at a 1:64 dilution. The serum-antigen suspension was incubated at 37°C for one hour followed by incubation at room temperature for 20 hours on a rocker. Thereafter, the suspension was centrifuged at $10,000 \times g$ for 11 minutes, saving the supernatant that was the absorbed serum.

Each serum was absorbed with the two antigens that yielded similar high titers in the previous IFA assay. Each of the absorbed sera was then tested by IFA to each of the antigens. If serum absorbed with one *Rickettsia* species showed no or minimal reaction against both antigens when absorbed with one antigen and a strong reaction to only one of the two antigens when absorbed with the second *Rickettsia* species, this serum was considered to contain antibodies stimulated by the *Rickettsia* species (or a very closely related species) that elicited the strong reaction in the second cross-absorption and had absorbed all the antibodies in the first reaction.

RESULTS

The IFA test detected antibodies reactive with *R. rickettsii* (titer ≥ 64) in 17 (77%) horses, 5 (31%) dogs, no donkeys and no humans (Table 1). The endpoint titer for each of the sera ranged from 64 to 4,096 (Table 2). When *R. rickettsii*-reactive sera were tested against four *Rickettsia* antigens (*R. rickettsii*, *R. africae*, *R. akari*, and *R. bellii*), five horse sera (H3, H4, H6, H71, H75) and one dog serum (D11) showed titers to *R. rickettsii* at least four-fold higher than to any of the other three antigens (Table 3). The antibody titers in these six

TABLE 1

Results of indirect immunofluorescence assay (IFA) for antibodies to *Rickettsia rickettsii* in humans and domestic animals from three farms in Pedreira Municipality, state of São Paulo, Brazil

Sera	Farm 1		Farm 2		Farm 3		Total	
	n*	IFA reactive sera† (%)	n*	IFA reactive sera† (%)	n*	IFA reactive sera† (%)	n*	IFA reactive sera† (%)
Humans	20	0 (0)	21	0 (0)	9	0 (0)	50	0 (0)
Horses	10	9 (90.0)	7	4 (57.1)	5	4 (80.0)	22	17 (77.3)
Donkeys	0	–	4	0 (0)	0	–	4	0 (0)
Dogs	4	1 (25.0)	6	4 (66.7)	6	0 (0)	16	5 (31.3)

* Total number of sera tested.

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

animals were considered to have been stimulated by *R. rickettsii*.

One horse serum (H9) exhibiting titers of 2,048 and 1,024 against *R. rickettsii* and *R. africae*, respectively, and another horse serum (H10) exhibiting titers of 4,096 and 2,048 against *R. rickettsii* and *R. africae*, respectively, were examined by cross-absorption. After absorption with *R. africae*, H9 and H10 demonstrated titers of 4,096 and 2,048, respectively, to *R. rickettsii* and no antibodies reactive with *R. africae*, but after absorption with *R. rickettsii*, there was no reaction against either of the two *Rickettsia* antigens. The antibody titers in these two horses were considered to have been stimulated by *R. rickettsii* (Table 3).

One dog serum (D7) exhibiting a titer of 1,024 against both *R. rickettsii* and *R. africae* was tested by cross-absorption. After absorption with *R. rickettsii*, dog serum D7 demonstrated a titer of 1,024 against *R. africae* and no antibodies against *R. rickettsii*, but after absorption with *R. africae*, there was no reaction against either of the two *Rickettsia* antigens. The antibody titers in this dog were considered to have been stimulated by a *Rickettsia* species very closely related to *R. africae*. Another dog serum (D10) exhibiting a titer of 512 against both *R. rickettsii* and *R. akari* was tested by cross-absorption. After absorption with *R. akari*, dog serum D10 demonstrated titers of 512 and 64 to *R. rickettsii* and *R. akari*, respectively, but after absorption with *R. rickettsii*, there was no reaction against *R. rickettsii* and a titer of 64 against *R. akari*. This serum was considered to contain antibodies stimulated by *R. rickettsii* (Table 3).

Overall, seven horse (H3, H4, H6, H9, H10, H71, H75) and two dog (D10, D11) sera were considered to contain antibodies stimulated by infection with *R. rickettsii*, and one dog serum (D7) was considered to contain antibodies stimulated by infection with a *Rickettsia* species very closely related to *R. africae* (Table 3). The *Rickettsia* species that stimulated the

TABLE 2

Endpoint antibody titers by indirect immunofluorescence assay (IFA) for *Rickettsia rickettsii* antigen in horses and dogs from Brazilian spotted fever–endemic areas in Pedreira Municipality, state of São Paulo, Brazil

Animal sera	Number of animals according to IFA titer							Total
	64	128	256	512	1,024	2,048	4,096	
Horses	–	1	2	4	7	2	1	17
Dogs	1	–	–	2	2	–	–	5

TABLE 3

Antibody titers by indirect immunofluorescence assay (IFA) for four *Rickettsia* species in horses (H) and dogs (D) from Pedreira Municipality, state of São Paulo, Brazil*

Animal sera	IFA titers for the following antigens				Probable antigen-stimulating antibody response
	<i>R. rickettsii</i>	<i>R. africae</i>	<i>R. akari</i>	<i>R. bellii</i>	
H3	1,024	256	128	256	<i>R. rickettsii</i>
H4	1,024	256	256	128	<i>R. rickettsii</i>
H6	1,024	128	64	128	<i>R. rickettsii</i>
H9	2,048	1,024	128	64	<i>R. rickettsii</i> †
H10	4,096	2,048	256	128	<i>R. rickettsii</i> †
H71	1,024	256	128	128	<i>R. rickettsii</i>
H72	2,048	1,024	64	NR	Undetermined‡
H75	1,024	256	128	64	<i>R. rickettsii</i>
D1	512	256	128	128	Undetermined
D7	1,024	1,024	256	256	Closely related to <i>R. africae</i> †§
D8	64	128	NR	128	Undetermined‡
D10	512	128	512	64	<i>R. rickettsii</i> †
D11	1,024	256	64	128	<i>R. rickettsii</i>

* NR = non-reactive at titer of 64 or higher.

† Determined by cross-absorption with the two antigens that yielded the highest titers.

‡ Cross-absorption was not performed.

§ Since *R. africae* has never been described in the area of the present study, we considered the homologous reaction to be related to some very closely related *Rickettsia* species.

antibodies in the sera of one horse (H72) and two dogs (D1, D8) could not be determined since these sera gave similar titers to more than one rickettsial species, but there was no more purified antigen available to perform cross-absorption tests.

DISCUSSION

The present study was conducted in a BSF-endemic area where at least four *Rickettsia* species have been reported: *R. rickettsii*, *R. felis*, *R. bellii*, and a *Rickettsia* species closely related to *R. africae* and *R. parkeri* and designated as strain COOPERI.⁶ Our serologic tests provided evidence that two of these *Rickettsia* species can infect domestic animals. One is *R. rickettsii*, which is the agent of BSF, and was considered to have elicited a homologous serologic response in seven horses and two dogs of the present study (Table 3). The second *Rickettsia* species is likely strain COOPERI, which is probably the agent that elicited an homologous serologic response in dog D7, which was demonstrated to have antibodies against a rickettsial species very closely related to *R. africae*. On the other hand, there was no evidence that *R. bellii* and *R. felis* caused infection in these dogs, horses, or humans. Interestingly, *R. felis* has been reported at high infection rates (>50%) in fleas (*Ctenocephalides felis felis*) collected on some dogs from farm 2 in the present study (Horta MC, Walker DH, unpublished data), whereas *R. bellii* has been reported in 40% of the *A. cooperi* ticks collected on all three farms of the present study.⁶

The tick *A. cajennense* is the main vector of *R. rickettsii* in Brazil. Horses, the primary host for all parasitic stages of this tick, are often massively infested by *A. cajennense* under natural conditions.²⁰ In situations of high tick burdens on horses, secondary hosts such as dogs and humans are often parasitized.²¹ These ecologic features have allowed an interpretation of the serologic reactivity of these animals and humans to *R. rickettsii* antigen in BSF-endemic areas where *A. cajennense* is the main vector. This pattern is characterized by a high fre-

quency of serologically positive horses, followed by a lower frequency in dogs, and an even lower frequency or absence of serologically positive humans. This pattern was observed in all three farms of the present study (Table 1) and in previous studies in other BSF-endemic areas in Brazil where *A. cajennense* is the main vector.^{12,13} These results suggest that horses can be used as sentinel animals for the detection of BSF-endemic areas, even before the occurrence of human cases of the disease. All human sera tested in the present study contained no antibodies to *R. rickettsii* antigens. This finding agrees with the fact that no humans of the present study had a history of BSF illness, although lethal cases of the disease have occurred on all three farms in the last few years. The high incidence of the *R. rickettsii* infection of horses can be explained by the fact that most of the *A. cajennense* population will feed on its primary hosts, which include horses. The lowest incidence (zero incidence) of *R. rickettsii* infection in humans can be explained by the lowest exposure of humans to tick infestation and also by the fact that humans tend to remove attached ticks before they can efficiently transmit bacteria via saliva.

Our failure to detect human exposure to rickettsial antigens could also be affected by the small sample size (50 humans). Other studies encompassing larger samples in BSF-endemic areas have shown small percentage of seropositive humans ($\approx 5\%$), when tested by IFA using *R. rickettsii* antigens.^{12,22} However, the antibodies were never shown to be directed against specific antigens of *R. rickettsii*. Since we have shown that at least three other *Rickettsia* species are also present in a BSF-endemic area, it is possible that some asymptomatic subjects with antibodies reactive with *R. rickettsii* had been infected by less pathogenic *Rickettsia* species. For this reason, there has been no convincing evidence to support asymptomatic human infection by *R. rickettsii*.

In the present study, there were seven horses and four donkeys grazing together in a same pasture in farm 2. Interestingly, all donkeys were serologically negative, in contrast to at least four (57%) serologically positive horses (Table 1). It is unclear if this result is related to the inability of *R. rickettsii* to infect donkeys or to a higher resistance of donkeys against infestation by *A. cajennense*, as shown in comparative studies with horses.²³

Our cross-absorption data support the presence of a second *Rickettsia* strain having infected the dog D7 from farm 2, a BSF-endemic area. This *Rickettsia* strain (COOPERI) is closely related to *R. africae* and *R. parkeri* and was recently reported infecting *A. cooperi* ticks from the farms 2 and 3 in the present study.⁶ More recently, we tested a serum collected on a sick dog from the farm 2 with fever, lethargy, anorexia, and muscle tenderness, which resulted in titers of 1,024, 256, 64, and 64 to *R. africae*, *R. rickettsii*, *R. akari*, and *R. bellii*, respectively (Labruna MB, Walker DH, unpublished data). This result reinforces the likelihood of strain COOPERI to infect dogs and also suggests that this rickettsial strain is pathogenic for dogs. Further studies in BSF-endemic areas where *A. cajennense* and *A. cooperi* are abundant should improve diagnostic methods to differentiate the *Rickettsia* species involved in the animal and human infections. Further studies should also try to isolate and establish strain COOPERI in the laboratory to perform additional taxonomic analysis and experimental infection of dogs, guinea pigs, and mice.

Received December 11, 2003. Accepted for publication February 9, 2004.

Acknowledgments: We thank Chao Hong and Donald Bouyer for technical support in the purification of *Rickettsia* antigen and Jere McBride for technical support in performing the cross-absorption test.

Financial support: This work was supported by the Fogarty International Center (grant D43TW00903 to David H. Walker and Marcelo B. Labruna), and Fundacao de Amparo a Pesquisa do Estado de São Paulo (grant 02/00644-0 to Marcelo B. Labruna and grant 00/02711-1 to Solange M. Gennari).

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