Rickettsia infection in five areas of the state of São Paulo, Brazil

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*This study investigated rickettsial infection in animals, humans, ticks, and fleas collected in five areas of the state of São Paulo. Eight flea species (*Adoratopsylla antiquorum antiquorum, Ctenocephalides felis felis, Polygenis atopus, Polygenis rimatus, Polygenis roberti roberti, Polygenis tripus, Rhopalopsyllus lugubris, *and* Rhopalopsyllus lutzi lutzi), *and five tick species (*Amblyomma aureolatum, Amblyomma cajennense, Amblyomma dubitatum, Ixodes loricatus, *and* Rhipicephalus sanguineus) *were collected from dogs, cats, and opossums.* Rickettsia felis *was the only rickettsia found infecting fleas, whereas* Rickettsia bellii *was the only agent infecting ticks, but no animal or human blood was shown to contain rickettsial DNA. Testing animal and human sera by indirect immunofluorescence assay against four rickettsia antigens (*R. rickettsii, R. parkeri, R. felis, *and* R. bellii), *some opossum, dog, horse, and human sera reacted to* R. rickettsii *with titers at least four-fold higher than to the other three rickettsial antigens. These sera were considered to have a predominant antibody response to* R. rickettsii. *Using the same criteria, opossum, dog, and horse sera showed predominant antibody response to* R. parkeri *or a very closely related genotype. Our serological results suggest that both* R. rickettsii *and* R. parkeri *infected animals and/or humans in the studied areas.*

Key words: *Rickettsia* - spotted fever - fleas - ticks - opossum - domestic animals

Rickettsiae are obligate intracellular bacteria that have been classically divided into two groups: the typhus group (TG), composed of *Rickettsia prowazekii* and *Rickettsia typhi,* which are associated with lice and fleas, respectively; and the spotted fever group (SFG), which includes more than 20 valid species, mostly associated with ticks (e.g., *Rickettsia rickettsii, Rickettsia parkeri*), and at least one species associated with fleas (*Rickettsia felis*). Other species, such as *Rickettsia bellii* and *Rickettsia canadensis,* both associated with ticks, have been considered a distinct group sharing antigenic, genetic and ecological characters with both TG and SFG rickettsiae (Parola et al. 2005b). Since all SFG *Rickettsia* share common outer membrane antigens, conventional serological assays employing a single *Rickettsia* antigen (e.g., *R. rickettsii*) allow detecting a SFG infection, but they do not identify the *Rickettsia* species responsible for the infection, even in the presence of high titers (La Scola & Raoult 1997). For a more specific serological diagnosis, it is recommended techniques such as testing serum against all known *Rickettsia* species present in a given area, or serum cross-absorption tests (La Scola & Raoult 1997, Parola et al. 2005b).

For many years, SFG rickettsiosis in the Americas was thought to be caused only by *R. rickettsii,* transmitted by ticks (Weiss & Moulder 1984). This rickettsia has been reported in several countries from North, Central, and South America causing an acute, highly lethal febrile disease receiving different names according to the country [e.g., Rocky Mountain spotted fever in the Unites States, Brazilian spotted fever (BSF) in Brazil] (Guedes et al. 2005). More recently, human spotted fever rickettsiosis due to *R. parkeri* have been reported in the United States (Paddock et al. 2004, Whitman et al. 2007). *R. parkeri* is also the most probably rickettsial agent of the Uruguayan spotted fever, a disease that has been reported in this country since early 1990s (Conti-Diaz 2003, Pacheco et al. 2006). While human infection by *R. parkeri* is yet to be documented in Brazil, two isolates of *R. parkeri* have been reported infecting *Amblyomma* ticks in the state of São Paulo (Labruna et al. 2004b, Silveira et al. 2007). In addition, serological evidence of canine infection due to *R. parkeri* has been reported in São Paulo and in northern Brazil (Horta et al. 2004a, Labruna et al. 2007a). *R. felis* is another human-pathogenic spotted fever *Rickettsia* reported in the Americas, including Brazil (Raoult et al. 2001). Unlike the above two *Rickettsia* species, which are vectored by ticks, *R. felis* is possibly carried by fleas especially *Ctenocephalides* spp. (Parola et al. 2005a).

There have been two reports of isolation of *R. rickettsii* from opossums (*Didelphis* spp.) from the states of São Paulo and Minas Gerais, Brazil (Moreira & Magalhães 1935, Travassos 1937). Interestingly, *R. rickettsii* has also been isolated from the North American opossum *Didelphis virginiana* (Bozeman et al.

1967). These findings led to several authors to suspect that opossums could participate in the natural cycle of *R. rickettsii,* serving as amplifier host for the agent (Dias & Martins 1939, Bozeman et al. 1967). More recently, *R. felis*-infected opossums (*D. virginiana*) were reported by serology in the USA, suggesting that opossums participate in the natural cycle of *R. felis* (Boostrom et al. 2002).

The present study investigates the presence of antibodies reactive to four *Rickettsia* species that have been recently described in the State of São Paulo (*R. rickettsii, R. parkeri, R. felis,* and *R. bellii*) among opossums, dogs, cats, horses, and humans from different areas of the state with or without history of recent cases of BSF. In addition, direct detection of *Rickettsia* DNA was attempted by molecular analyses of animal blood samples and from ticks and fleas collected on opossums, dogs and cats.

MATERIALS AND METHODS

Study areas - This study was conducted in four areas in the state of São Paulo with history of recent confirmed cases of BSF in humans (endemic areas), and a fifth area with no history of BSF cases (nonendemic area) (Figure).

Endemic area 1: Pedreira (22º44′55.7″S, 46º53′55.7″W). 41 confirmed BSF cases of have been reported since 1985 (CVE 2006). The clinical severity (including high lethality), serological analyses, and isolation of *Rickettsia* from clinical samples of these cases were compatible with *R. rickettsii* (Lemos et al. 2001, Angerami et al. 2006). The presence of *R. bellii* and *R. parkeri* (strain Cooperi) was reported infecting the capybara tick *Amblyomma dubitatum* (Labruna et al. 2004b), whereas *R. felis* was reported infecting fleas *Ctenocephalides felis felis* (Horta et al. 2005). Serological analyses employing different rickettsia antigens and serum cross-absorption tests have indicated infection by *R. rickettsii* in horses and dogs, and by *Rickettsia* spp (possibly *R. parkeri*) in one dog (Horta et al. 2004a). In addition, serological analysis has indicated SFG infection in hu-

mans, horses, and dogs (Lemos et al. 1996a); and a SFG rickettsia has been isolated from an *A. dubitatum* tick (Lemos et al. 1996b). Tick species incriminated as vector of BSF in this area has been *Amblyomma cajennense*, although *A. dubitatum* has also been found in high population density. Both tick species are sustained by large populations of free-living capybaras (*Hydrochaeris hydrochaeris*); but horses are also main hosts for *A. cajennense* (Labruna et al. 2004b, Sangioni et al. 2005). Samples were collected in the same farms sampled by Horta et al. (2004a) and in other nearby farms.

Endemic area 2: Mogi das Cruzes, district of Taiaçupeba (23º38′34.5″S, 46º11′3.5″W). Several cases of BSF were reported in this municipality from 1957 to 1974 (Tiriba et al. 1968); nine confirmed cases of BSF were reported from 1998 to 2006 (CVE 2006). The clinical severity and high lethality (≈80%) of these cases are compatible with *R. rickettsii* (Fontes et al. 2000). In one case, *R. rickettsii* was isolated by inoculation of clinical samples in guinea pigs (Melles et al. 1992). Both *R. rickettsii* and *R. bellii* have been isolated from ticks, and *A. aureolatum* was incriminated as the vector of BSF (Pinter & Labruna 2006). In addition, *R. felis* has been reported in fleas *C. felis felis* (Horta et al. 2005). Populations of *A. aureolatum* are sustained mainly by dogs (hosts for adult ticks) and small wild animals (rodents and birds). Capybaras, horses, as well as *A. cajennense* and *A. dubitatum* are rarely found in this area (Adriano Pinter et al. unpublished observations). Samples were collected in the Taiaçupeba district, the same area sampled by Pinter and Labruna (2006).

Endemic area 3: Piracicaba (22º42′22.8″S, 47º37′39.4″W). A total of 20 BSF-human cases have been reported in this area since 2002, but only a few of them were laboratory-confirmed (CVE 2006). Some of these cases were lethal, clinically compatible with infection due to *R. rickettsii* (CVE 2006). The tick species incriminated as vector of BSF is *A. cajennense.* The capybara tick *A. dubitatum* occurs in low frequency, and has been found infected by *R. bellii* (Horta et al. 2004b). Both tick species have been sustained by large populations of free-living capybaras; but horses (less abundant) may also play a role in sustaining *A. cajennense* populations. Samples were collected in the University of São Paulo campus of Piracicaba.

Endemic area 4: São Paulo (23º35′15.8″S, 46º39′28.1″W): BSF has been reported in different areas of this large municipality since the end of the 1920s (Gomes 1932, Monteiro & Tiriba 1976). During this period, several isolates from humans and two from opossums obtained through guinea pig inoculation showed to be compatible with *R. rickettsii* (Gomes 1932, Travassos 1937, Tiriba et al. 1968, Monteiro & Tiriba 1976). Recently (2002 to 2006), five laboratory-confirmed cases heve been reported (CVE 2006). A *R. rickettsii*-infected *A. aureolatum* tick was reported in this area decades ago (Gomes 1933), which remains as the possible tick vector. Currently, both *A. cajennense* and *A. aureolatum* are found in different parts of São Paulo municipality (Vieira et al. 2004). Samples were collected in University of São Paulo campus of São Paulo, Ibirapuera Park, and SESC-Interlagos.

Geographic location of the five areas of the present study in the state of São Paulo, Brazil.

Nonendemic area: Pirassununga (21º56´56.1"S, 47º27´16.9"W). BSF has never been notified or confirmed in this area. Similarly, no *Rickettsia*-infected tick or flea has been reported. On the other hand, there are very large populations of *A. cajennense* ticks sustained mainly by horses (Sangioni et al. 2005), and by capybaras in some particular patches. Capybaras also sustains some smaller populations of *A. dubitatum*. Samples were collected in the University of São Paulo campus of Pirassununga.

Animal and human samples - Capture of free-living opossums was performed from May 2003 to February 2005. In each area, a total of 15-25 work nights were necessary for collection of at least12 opossums per area. The minimum of 12 opossums per area was determined by numerical expectation based on epidemiological studies employing opossum captures (Cáceres & Monteiro Filho 2001). Captured opossums were anesthetized with ketamine 15-30 mg/kg for collection of blood and ectoparasites (fleas and ticks). Each trapped opossum was ear marked before releasing in the capture site, in order to identify possible recapture. Blood samples were collected from all dogs, cats, horses, and humans that used to live or work in the study areas. Serum was individually obtained by centrifugation, and stored at -20ºC for indirect immunofluorescence assay (IFA), and blood clot was stored at -80ºC for molecular analysis. Fleas and ticks were collected from all dogs and cats. This study was submitted and approved by the Ethical Committees of the University of São Paulo (protocol FMVZ 301/2003, ICB 495/CEP/2003), and by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis - IBAMA (02027.023912/2002).

Fleas and adult ticks were immediately preserved in 100% ethanol. Due to the impossibility of a precise identification of subadult ticks, captured larvae and nymphs were reared until the adult stage by feeding on laboratory naïve hosts (rabbits *Oryctolagus cuniculus* or guinea pigs *Cavia porcellus*), and subsequently identified to species according to Barros-Battesti et al. (2006). Ticks that died before reaching the adult stage were identified just to the generic level. Fleas were identified according to Linardi and Guimarães (2000)

IFA was used to detect human and animal serum antibodies reactive to *Rickettsia* spp. Glass slides were prepared according to Horta et al. (2004a), employing four *Rickettsia* species that are routinely maintained in our laboratory: *R. rickettsii* strain Taiaçu (Pinter & Labruna 2006), *R. parkeri* strain São Paulo (Silveira et al. 2007), *R. felis* strain Pedreira (Horta et al. 2006), and *R. bellii* strain Mogi (Pinter & Labruna 2006). Each *Rickettsia* strain was cultivated in Vero cells (or C6/36 cells in case of *R. felis*), and harvested when nearly 100% of the cells were infected. The infected cells were centrifuged at 4,000 *g* for 10 min, and 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 0.1% sodium azide. Ten microliters of rickettsiae-infected cells were applied onto each of 12 wells on microscopic slides, air-dried, fixed in acetone for 10 min, and stored at -80ºC 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 min in a humid chamber. The slides were rinsed once, and then washed twice for 15 min per wash in PBS. The slides were incubated with fluorescein isothiocyanate-labeled sheep anti-opossum IgG (CCZ, São Paulo, Brazil), goat anti-human IgG, goat anti-horse IgG, goat anti-cat IgG, or rabbit anti-dog IgG (Sigma, St Louis, USA), and washed as described earlier. The slides were mounted with buffered glycerin under coverslips. The slides were read using an ultraviolet microscope (Olympus, Tokyo, Japan) at 400x magnification. Serum was considered to contain antibodies against the rickettsiae if it displayed a reaction at the 1:64 dilution. End-point titers against each *Rickettsia* strain were determined by testing serial two-fold serum dilutions. Reactive sera were tested in two or three replications before determining the endpoint titer. In each slide, a serum previously shown to be non-reactive (negative control) and a known reactive serum (positive control) were tested. Serum showing for a *Rickettsia* species titer at least four-fold higher than that observed for any other *Rickettsia* species was considered to elicit a predominant antibody response to the first *Rickettsia* species or to a very closely related genotype (Horta et al. 2004a, Labruna et al. 2007a).

DNA extraction was performed individually on whole fleas, whole subadult ticks, or on extirpated legs from adult ticks. Each flea or tick or a pool of legs from a single adult tick was ground in 50 µl of TE buffer (10mM Tris HCl; 1 mM ethylene diamine tetracetic acid pH 8.0) in sterile micro tubes. The final suspension was boiled at 100ºC for 20 min and held at -20ºC until tested by polymerase chain reaction (PCR). DNA extraction of animal and human blood samples were performed using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), starting with 200 ml of blood clot from each individual sample. Fleas and ticks were individually processed by PCR using primers CS-78 (5'-GCAAGTATCG-GTGAGGATGTAAT-3') and CS-323 (5'-GCTTCCTTA-AAATTCAATAAATCAGGAT-3'), which amplify a 401-bp fragment of the citrate sinthase gene (*gltA*), possibly present in all *Rickettsia* species (Labruna et al. 2004b). For blood samples, primers CS-5 (5'-GAGAGAAAAT-TATATCCAAATGTTGAT-3') and CS-6 (AGGGTCTT-CGTGCATTTCTT), which amplify a 147-bp fragment of the rickettsial *gltA* gene were used. These later primers have shown sensitivity down to a single copy of *R. rickettsii* and 10 copies of *R. bellii* (Labruna et al. 2004b). For each reaction, 5 µl of the DNA template from each individual sample (flea, tick, animal, or human) were added to 5 µl of PCR buffer (10X), 8 µl of deoxynucleotide triphosphates mixture (1.25 mM), 1.5 µl MgCl$_2$ (50 mM), 25 pmol of each primer, 0.25 µl of *Taq* polymerase (5,000 U/ml) and bi-distilled water to a final volume of 50 µl. For each reaction, DNA extracted from *R. parkeri*-infected *A. cajennense* ticks was used as positive control (Sangioni et al. 2005) and at least three aliquots of bi-distilled water were used as negative controls. PCR thermal conditions were used as previously described (Labruna et al. 2004b). PCR products were stained by ethidium bromide and visualized by electrophoresis in 1.5% agarose gel.

All PCR products of the expected amplicon size were purified, and their forward and reverse nucleotide sequences were determined in a DNA sequencer analyzer (ABI Prism 3100 Genetic – Applied Biosystems/Perking Elmer, California, USA). Partial sequences obtained were submitted to BLAST (Basic Local Alignment Search Tool) analysis (Altschl et al. 1990) to determine similarities to other *Rickettsia* species.

RESULTS

A total of 94 opossums (65 *D. aurita* and 29 *D. albiventris*) were trapped in BSF-endemic and nonendemic areas. In Pedreira, Mogi das Cruzes, and São Paulo (endemic areas 1, 2 and 4, respectively), all opossums were *D. aurita,* whereas *D. albiventris* comprised all opossums from Piracicaba (endemic area 3) and Pirassununga (non-endemic area). A total of 55 dogs, 25 cats, 85 horses, and 229 humans were sampled. The numbers of animals and humans sampled in each of the five areas are shown in Table I.

IFA - Overall, sera from 64 (68.1%) opossums (43 *D. aurita* and 21 *D. albiventris*), 33 dogs (60.0%), 21 cats (84.0%), 62 horses (72.9%), and 34 humans (14.3%) reacted positively (titer > 64) to at least one of the four *Rickettsia* antigens tested (Table I). In Pedreira, Mogi das Cruzes, Piracicaba, and São Paulo (endemic areas 1,

2, 3, and 4, respectively), at least three, two, four, and one opossum sera (respectively) showed titers to *R. rickettsii* at least four-fold higher than to any of the other three rickettsial antigens. These serum antibody responses were considered to be produced against to *R. rickettsii* or a very closely related genotype. Using the same criteria, antibody responses in sera from two dogs from Pedreira and two dogs from Mogi das Cruzes, one horse and one human from Piracicaba, and one human from Pedreira were also considered to be produced to *R. rickettsii* (Table II). Additionally, one opossum from Mogi das Cruzes, one dog and one horse from Piracicaba, and one opossum from Pedreira showed antibody responses that were considered to be produced to *R. parkeri*. Regarding Pirassununga (nonendemic area), only a single opossum serum showed antibody response that was considered to be produced to *R. parkeri* or a very closely related genotype. Despite of several sera also reacted positively to *R. felis* and *R. bellii,* none antibody response could be considered to be produced to these agents due to similar or low titers in comparison with titers to *R. rickettsii* or *R. parkeri.*

Fleas and Ticks - A total of 312 fleas were collected from opossums. Overall, the fleas *C. felis felis, Polygenis (Neopolygenis) atopus, and Rhopalopsyllus lutzi lutzi* comprised 94.5% of the collected fleas. Other five spe-

TABLE I

Results of indirect immunofluorescence assay for antibodies reactive with four *Rickettsia* species in opossums, domestic animals, and humans from five areas in the state of São Paulo, Brazil

Sera	Area[a] (No. of individuals)	No. of reactive sera (% reactive sera)			
		R. rickettsii	*R. parkeri*	*R. felis*	*R. bellii*
Opossum	Pedreira (14)	11 (78.6)	10 (71.4)	9 (64.3)	2 (14.3)
	Mogi das Cruzes (16)	10 (62.5)	8 (50.0)	3 (18.7)	0 (0)
	Piracicaba (17)	11 (64.7)	12 (70.6)	11 (64.7)	6 (35.3)
	São Paulo (35)	18 (51.4)	19 (54.3)	6 (17.1)	6 (17.1)
	Pirassununga (12)	6 (50.0)	6 (50.0)	4 (33.3)	1 (8.3)
Dog	Pedreira (19)	11 (57.9)	12 (63.1)	13 (68.4)	7 (36.8)
	Mogi das Cruzes (13)	9 (69.2)	9 (69.2)	8 (61.5)	5 (38.5)
	Piracicaba (7)	3 (42.8)	3 (42.8)	2 (28.6)	1 (14.2)
	São Paulo (11)	3 (27.3)	3 (27.3)	3 (27.3)	0 (0)
	Pirassununga (5)	1 (20.0)	0 (0)	0 (0)	0 (0)
Cat	Pedreira (3)	3 (100)	3 (100)	3 (100)	2 (66,7)
	Mogi das Cruzes (3)	3 (100)	3 (100)	3 (100)	2 (66,7)
	Piracicaba (7)	7 (100)	6 (85.7)	6 (85.7)	4 (57.1)
	São Paulo (6)	3 (50.0)	3 (50.0)	2 (33.3)	0 (0)
	Pirassununga (6)	3 (50.0)	4 (66.7)	2 (33.3)	3 (50.0)
Horse	Pedreira (20)	18 (90.5)	18 (90.0)	19 (95.0)	19 (95.0)
	Mogi das Cruzes (5)	2 (40.0)	2 (40.0)	2 (40.0)	3 (60.0)
	Piracicaba (20)	17 (85.0)	17 (85.0)	17 (85.0)	19 (95.0)
	São Paulo (19)	7 (36.8)	7 (36.8)	7 (36.8)	11 (57.9)
	Pirassununga (21)	4 (19.0)	4 (19.0)	4 (19.0)	10 (47.6)
Human	Pedreira (51)	8 (15.7)	8 (15.7)	8 (15.7)	5 (8.8)
	Mogi das Cruzes (21)	4 (19.0)	4 (19.0)	4 (19.0)	4 (19.0)
	Piracicaba (69)	7 (10.1)	5 (7.2)	5 (7.2)	6 (8.7)
	São Paulo (68)	10 (14.7)	10 (14.7)	6 (8.8)	5 (7.3)
	Pirassununga (28)	5 (17.8)	5 (17.8)	5 (17.8)	3 (10.7)

a: Pedreira, Mogi das Cruzes, Piracicaba, São Paulo (endemic areas), and Pirassununga (nonendemic area).

TABLE II

Antibody titers by indirect immunofluorescence assay (IFA) for four *Rickettsia* species in opossums (O), dogs (D), horses (H) and humans (HU) from five areas in the state of São Paulo. Predominant antibody response to antigen (PARA) was determined for the *Rickettsia* species that elicited titer at least four fold higher than titers elicited by the other *Rickettsia* species

| Sera | Area | IFA titers for the following antigens | | | | PARA |
		R. rickettsii	*R. parkeri*	*R. felis*	*R. bellii*	
O-29	Piracicaba	512	128	64	NR	*R. rickettsii*
O-30	Piracicaba	32.768	4.096	2.048	4.096	*R. rickettsii*
O-33	Piracicaba	4.096	512	512	1.024	*R. rickettsii*
O-36	São Paulo	1.024	256	256	NR	*R. rickettsii*
O-49	Pedreira	2.048	256	128	NR	*R. rickettsii*
O-55	Piracicaba	512	128	128	128	*R. rickettsii*
O-56	Mogi das Cruzes	2.048	256	512	NR	*R. rickettsii*
O-70	Mogi das Cruzes	256	64	NR	NR	*R. rickettsii*
O-93	Pedreira	2.048	512	64	NR	*R. rickettsii*
O-96	Pedreira	512	64	128	NR	*R. rickettsii*
O-46	Pirassununga	256	1.024	256	NR	*R. parkeri*
O-68	Pedreira	64	256	64	NR	*R. parkeri*
O-71	Mogi das Cruzes	64	256	NR	NR	*R. parkeri*
D-27	Pedreira	4.096	1.024	1.024	128	*R. rickettsii*
D-29	Pedreira	2.048	512	256	64	*R. rickettsii*
D-33	Mogi das Cruzes	256	64	64	NR	*R. rickettsii*
D-36	Mogi das Cruzes	2.048	512	NR	NR	*R. rickettsii*
D-51	Piracicaba	64	256	NR	NR	*R. parkeri*
H-7	Piracicaba	1.024	256	128	256	*R. rickettsii*
H-20	Piracicaba	1.024	4.096	512	512	*R. parkeri*
HU-75	Pedreira	256	64	64	NR	*R. rickettsii*
HU-29	Piracicaba	512	128	64	128	*R. rickettsii*

a: Pedreira, Mogi das Cruzes, Piracicaba, São Paulo (endemic areas), and Pirassununga (nonendemic area). NR: non reactive at titer 64 or higher.

cies [*Polygenis (Polygenis) roberti roberti, P. (Polygenis) tripus, P. (Polygenis) rimatus, Rhopalopsyllus lugubris,* and *Adoratopsylla (Adorato-psylla) antiquorum antiquorum*)] comprised 5.5% of the collected fleas. *C. felis felis* was the most common flea species infesting opossums in the four endemic areas. However, it was absent from Pirassununga (nonendemic area), where *P. atopus* and *R. lutzi lutzi* were the most prevalent flea species. A total of 278 fleas were collected from dogs (n = 212 fleas) and cats (n = 66). All fleas from dogs were identified as *C. felis felis,* whereas fleas from cats were identified as *C. felis felis* (n = 59) and *R. lutzi lutzi* (n = 7). No flea was collected from horses or humans.

R. felis was the only *Rickettsia* species found infecting fleas in all five areas. Infection rates varied from 33.3 to 72.7% among *C. felis felis* fleas from opossums (Table III), from 13.5 to 52.7% among *C. felis felis* fleas from dogs, and from 29.4 to 83.3% among *C. felis felis* fleas from cats (Table IV). In addition, 3.3 and 8.0% of the *P. atopus* fleas collected on opossums from Mogi das Cruzes and Pirassununga, respectively, were also infected by *R. felis.* Additional flea species collected from opossums and that did not contain rickettsial DNA were *R. lutzi lutzi* (Pedreira, n = 16; Piracicaba, 12; Pirassununga, 24), *P. roberti roberti* (Mogi das Cruzes, 7; Pirassununga, 1), *P. tripus* (Mogi das Cruzes, 2; Piracicaba, 1; Pirassununga, 1), *R. lugubris* (Pedreira, 3), *A. antiquorum antiquorum* (Mogi das Cruzes, 1), and *P. rimatus* (Mogi das Cruzes, 1).

A total of 709 tick specimens were collected from opossums, whereas 115 and 10 ticks were collected from dogs and cats, respectively. Ticks were found infesting animals from all studied areas, except for dogs, and cats from Pirassununga, and cats from São Paulo. Three tick species were found infesting opossums (*A. cajennense* in Pedreira and Piracicaba; *A. dubitatum* in Mogi das Cruzes, Piracicaba and Pirassununga; and *Ixodes loricatus* in Mogi das Cruzes, São Paulo and Pirassununga), whereas three tick species were found infesting dogs and cats (*Rhipicephalus sanguineus* and *A. cajennense* in Pedreira and Piracicaba; and *A. aureolatum* in Mogi das Cruzes and São Paulo). Ticks were not collected on horses, although infestations by both *A. cajennense* and *Anocentor nitens* ticks were visually detected in most of the horses from Pedreira, Piracicaba, and Pirassununga. No tick was collected from humans, although humans complained about suffering from frequent tick infestation in all areas, except for Mogi das Cruzes and São Paulo, where tick infestation was occasionally noticed.

All ticks collected on opossums, dogs and cats were tested by PCR. No *Rickettsia* was detected in ticks from dogs and cats, whereas *R. bellii* was the only species detected in ticks (*I. loricatus, A. dubitatum,* and *Amblyomma* sp) from opossums (Table III). No rickettsial DNA was detected in animal or human blood samples.

TABLE III

Fleas and ticks collected on opossums and *Rickettsia* infection determined by PCR and DNA sequencing,
in five areas in the state of São Paulo

| | Fleas[b] | | Ticks[c] | | | | |
| | *Ctenocephalides felis felis* | *Polygenis (N.) atopus* | *Amblyomma dubitatum* | | *Amblyomma* spp | | *Ixodes loricatus* |
Area[a]	Adult	Adult	Nymph	Larva	Nymph	Larva	Adult
Pedreira	19/37 (51.3)	0/1 (0)	-	-	1/35 (2.8)	0/1 (0)	-
Mogi das Cruzes	12/36 (33.3)	2/61 (3.3)	1/3 (33.3)	0/9 (0)	0/3 (0)	0/21 (0)	38/73 (52.0)
Piracicaba	8/11 (72.7)	-	1/2 (50)	0/7 (0)	1/254 (0.4)	3/136 (2.2)	-
São Paulo	27/60 (45.0)	-	-	-	-	-	6/6 (100)
Pirassununga	-	3/37 (8.1)	-	0/2 (0)	0/26 (0)	0/19 (0)	23/31 (74.2)
Total	66/144 (45.8)	5/99 (5.0)	2/5 (40)	0/18 (0)	2/318 (0.6)	3/177 (1.7)	67/110 (60.9)

a: Pedreira, Mogi das Cruzes, Piracicaba, São Paulo (endemic areas), and Pirassununga (nonendemic area); *b*: values presented as number of *R. felis*-infected fleas/number of collected fleas (% infected fleas); *c:* values presented as number of *R. bellii*-infected ticks/number of collected ticks (% infected ticks).

TABLE IV

Fleas collected on dogs and cats and *R. felis* infection determined by PCR and DNA sequencing,
in six areas in the state of São Paulo

| | Fleas from dogs | Fleas from cats | | |
Area[a]	*C. felis felis*[b]	*C. felis felis*[b]	*R. lutzi lutzi*[b]	Total[b]
Pedreira	39/74 (52.7)	5/6 (83.3)	0/1 (0)	44/81 (54.3)
Mogi das Cruzes	10/25 (40.0)	3/6 (50.0)	-	13/31 (41.9)
Piracicaba	21/46 (45.6)	3/6 (50.0)	0/1 (0)	24/53 (45.3)
São Paulo	14/30 (46.7)	5/17 (29.4)	-	19/47 (40.4)
Pirassununga	5/37 (13.5)	9/24 (37.5)	0/5 (0)	14/66 (21.2)
Total	89/212 (42.0)	25/59 (42.4)	0/7 (0)	114/278 (41.0)

a: Pedreira, Mogi das Cruzes, Piracicaba, São Paulo (endemic areas), and Pirassununga (nonendemic area); *b*: values presented as number of *R. felis*-infected fleas/ number of collected fleas (% infected fleas).

DISCUSSION

BSF is thought to be caused by *R. rickettsii,* however the vast majority of the laboratory-confirmed cases in the state of São Paulo have been diagnosed solely by serology employing a single antigen (*R. rickettsii*) or/ and isolation of the agent in cell culture without further species identification, and/or PCR without DNA sequencing (Nascimento & Schumaker 2004). Technically, these diagnostic procedures have not allowed a precise identification of the rickettsial agent; at best, defining SFG rickettsiosis, since the three employed techniques could be efficient for any spotted fever group *Rickettsia* species. During the last six years, four SFG species other than *R. rickettsii* were reported in Brazil, two of them recognized as agents of spotted fever rickettsiosis in humans (*R. parkeri* and *R. felis*) (Raoult et al. 2001, Labruna et al. 2004a, b, Horta et al. 2005, Labruna et al. 2007c, Silveira et al. 2007). Thus, it is possible that some of the BSF cases diagnosed in the state of São Paulo could be caused by other SFG rickettsia, such as *R. parkeri* or *R. felis.*

The strong serological reactivity with *R. rickettsii* is an evidence that this species might be inducing an infection in humans and/or animals from the four endemic areas, whereas no convincing evidence of infection due to this agent was found in Pirassununga, the nonendemic area (Table II). On the other hand, serological reactivity for *R. parkeri* was detected in animals from four out of the five studied areas, including the nonendemic area. Since *R. parkeri* spotted fever is a newly identified disease**,** which is less virulent (no lethality has been reported), and less widely recognized than the infection caused by *R. rickettsii,* it is quite possible that human-*R. parkeri* spotted fever has been occurring without a proper diagnosis in Pedreira, Mogi das Cruzes, Piracicaba, and Pirassununga. A potential tick vector of *R. parkeri* in these areas is the capybara tick *A. dubitatum*, which has been recently found infected by *R. parkeri* (strain Cooperi) in Pedreira, exactly in the same site sampled in the present study (Labruna et al. 2004b), and where a *R. parkeri* infection was reported in one dog by both IFA and cross-absorption technique (Horta et al. 2004a).

In addition, successful experimental infection of *R. parkeri* has been achieved in *A. cajennense* (Sangioni et al. 2005), which should also be considered a potential vector.

R. felis is a spotted fever group rickettsia that has been proved to be pathogenic for humans in several countries, including Brazil (Raoult et al. 2001). However, we found no clear serological reactivity of animal or human-*R. felis* infection in the present study. This finding was surprising, since *R. felis* was detected in high frequencies among the most common flea species from opossums, dogs and cats in the five studied areas. In the USA, Boostrom et al. (2002) reported *R. felis*-infected opossums (*D. virginiana*) by means of serology. However, these authors did not find correlation between seropositive opossums and *R. felis*-infected fleas collected on these opossums. After its first report infecting *C. felis felis* fleas in the USA (Adams et al. 1990), *R. felis* has turned to be a cosmopolitan species infecting mostly *C. felis* fleas (Parola et al. 2005a). The contrast of few reported human cases of *R. felis*-spotted fever with the cosmopolitan distribution of *R. felis*-infected fleas deserves further investigations.

No clear reactivity of *R. bellii* infection in humans or animals was found in the present study. *R. bellii* has been the most frequent rickettsia species infecting ticks in Brazil, being reported in at least nine tick species (Labruna et al. 2007c). In contrast to the present study, there has been substantial serological evidence that *R. bellii* produces infection in capybaras, which are often parasitized by *A. dubitatum* (Pacheco et al. 2007). This tick species has been often found infected by *R. bellii* in the state of São Paulo (Labruna et al. 2004b, Horta et al. 2004b).

In a recent study with horses, dogs, and humans in Pedreira, Sangioni et al. (2005) recommended surveys of horse sera (and dogs in a lesser extent) as a useful method for BSF surveillance in areas where humans are exposed to *A. cajennense* ticks. This recommendation is based on the fact that horses are primary hosts for all parasitic stages of *A. cajennense*, a tick that also attacks dogs and other medium-sized mammals. Our results showed that horses were in fact excellent sentinels for BSF in Pedreira and Piracicaba, two areas where *A. cajennense* is incriminated as the main vector of *R. rickettsii*. In these two areas, 85 to 90% of the horses were seropositive for *R. rickettsii*. On the other hand, much lower frequencies (19 - 40%) of seropositive horses for *R. rickettsii* were detected in Mogi das Cruzes, São Paulo, and Pirassununga. In Mogi das Cruzes, *A. aureolatum* is the primary vector of *R. rickettsii*, corroborating our finding of 69.2% of seropositive dogs, which are primary hosts for the adult stage of *A. aureolatum*. In São Paulo and Pirassununga, neither horses nor dogs were demonstrated to be good sentinels for BSF or *R. parkeri* infection; in these two areas, cats and opossums showed the highest seropositivities for *R. rickettsii* and *R. parkeri*. In addition, cats and opossums were also excellent sentinels for these two agents in Pedreira, Mogi das Cruzes, and Piracicaba. These results indicate that surveys of cats and opossums sera would be more efficient than horse or dog for BSF surveillance. This superiority of cats and opossums is pos-

sibly related to the fact that they are more exposed to ticks than the other animals. Opossums are wild animals with synantropic habits. Rural cats are domestic animals with wild habits; thus they also live in both wild and domestic environments. In contrast, horses are usually restricted to pastures while rural dog habits are high variable, ranging from completely domestic to those with some wild habits.

Comparing to other animals, human sera demonstrated the lowest frequencies of seropositivity for *Rickettsia* spp. This result was expected since humans are much less exposed to ticks than other animals. Only two human sera were considered homologous to *R. rickettsii*. Several other human sera reacted positively to *Rickettsia* spp., but it was not possible to determine the *Rickettsia* species involved in a predominant antibody response. Based on the results obtained with animals, it is possible that some of these humans were infected by *R. rickettsii* or *R. parkeri*; also, infection by *R. felis* could not be ruled out, since sera showed similar titers (< 4 fold difference) for these three agents (data not shown).

Our molecular analysis showed that 13.5 - 83.3% of the *C. felis felis* fleas collected from the five areas of the present study were infected with *R. felis*. In addition, 3.3 and 8.1% of the *P. atopus* fleas collected on opossums from Mogi das Cruzes and Pirassununga, respectively, were also infected by *R. felis*. This is the first report of this rickettsia infecting this wild flea species, indigenous from the Neotropical region. Interestingly, in Pirassununga, there was no *C. felis felis* infesting opossums. This result suggests that *P. atopus* is capable of maintaining the infection by *R. felis* without the presence of *C. felis felis*.

It is noteworthy our findings of *A. dubitatum* nymphs parasitizing opossums for the first time. This tick species uses capybaras as primary hosts for all parasitic stages. However, larvae and nymphs seems to have a much broader host range, including humans (Labruna et al. 2007b). Five tick species collected from opossums, dogs, and cats were tested for rickettsial infection. No rickettsia was found infecting ticks from dogs and cats, while only *R. bellii* was detected in two species of ticks from opossums (*A. dubitatum* and *I. loricatus*). Previously, *R. bellii* was reported infecting these two tick species in the state of São Paulo (Labruna et al. 2004b, Horta et al. 2004b). Previous studies in the state of São Paulo reported *R. rickettsii* in *A. aureolatum* (Gomes 1933, Pinter & Labruna 2006) and *A. cajennense* (Vallejo-Freire 1946) ticks, and *R. parkeri* (strain COOPERI) in *A. dubitatum* ticks (Labruna et al. 2004b). However, none of these agents were found infecting ticks in the present study. The reasons for these negative results are possibly the small number of tested ticks, and the low infection rates of *R. rickettsii* and *R. parkeri* that are usually reported for *A. aureolatum, A. cajennense,* or *A. dubitatum* (Labruna et al. 2004b, Sangioni et al. 2005, Pinter & Labruna 2006)

We did not detect rickettsial DNA in any human and animal blood samples in the present study. Detection of rickettsia in vertebrate blood is usually a rare event, since once infected, vertebrates display a ricketsemia for only

a few days or weeks, and thereafter no rickettsia is found in the blood (Burgdorfer et al. 1988). In addition, *Rickettsia* spp. are known to infect endothelial cells in vertebrates, thus its concentration in the blood is very low, incapable of be detected by molecular analysis (La Scola & Raoult 1997).

Previously, *R. rickettsii* was identified infecting humans from Pedreira, Mogi das Cruzes, and São Paulo. The present study presents serological reactivity of *R. rickettsii* infection in humans from Piracicaba. Previous serological diagnosis of BSF in this area did not allow a precise identification of the agent, although it was clearly a spotted fever disease clinically and epidemiologically compatible with *R. rickettsii* infection. Despite of the presence of at least four *Rickettsia* species in most of the studied areas, there was serological evidence of rickettsial infection in humans and/or animals by only two species: *R. rickettsii* and *R. parkeri*. These findings strongly suggest that some of the BSF cases occurring in BSF-endemic areas of the state of São Paulo could be caused by *R. parkeri*. In addition, our results showed that *R. parkeri* could also be present in areas considered non-endemic for BSF. Thus, there is a possibility that the distribution area of *R. parkeri* is much broader in the state of São Paulo, since one of its main tick hosts (the capybara tick, *A. dubitatum*) is widely distributed (Vieira et al. 2004). Finally, since *R. rickettsii* has been the sole antigen regularly employed for serological diagnosis of BSF, it is likely that human spotted fever cases due to *R. parkeri* or other spotted fever group rickettsiae are being misidentified as BSF in Brazil.

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