Survey for Tick-Borne Zoonoses in the State of Espírito Santo, Southeastern Brazil

Mariana G. Spolidorio,* Marcelo B. Labruna, Rosângela Z. Machado, Jonas Moraes-Filho, Augusto M. Zago, Dirlei M. Donatelle, Sônia R. Pinheiro, Iara Silveira, Késia M. Caliarí, and Natalino H. Yoshinari

Laboratório de Investigação Médica 17 (LIM17), Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil; Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, Brazil; Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Julio de Mesquita Filho,” Jaboaticabal, Brazil; Núcleo de Vigilância em Saúde, Superintendência Regional de Saúde de Colatina, Colatina, Espírito Santo, Brazil; Centro Universitário São Camilo Espírito Santo, Cachoeiro de Itapemirim, Espírito Santo, Brazil

Abstract. Blood samples collected from 201 humans, 92 dogs, and 27 horses in the state of Espírito Santo, Brazil, were tested by polymerase chain reaction, indirect immunofluorescence assays, and indirect enzyme-linked immunosorbent assay for tick-borne diseases (rickettsiosis, ehrlichiosis, anaplasmosis, borreliosis, babesiosis). Our results indicated that the surveyed counties are endemic for spotted fever group rickettsiosis because sera from 70 (34.8%) humans, 7 (7.6%) dogs, and 7 (25.9%) horses were reactive to at least one of the six *Rickettsia* species tested. Although there was evidence of ehrlichiosis (*Ehrlichia canis*) and babesiosis (*Babesia canis vogeli*, *Theileria equi*) in domestic animals, no human was positive for babesiosis and only four individuals were serologically positive for *E. canis*. *Borrelia burgdorferi*-serologic reactive sera were rare among humans and horses, but encompassed 51% of the canine samples, suggesting that dogs and their ticks can be part of the epidemiological cycle of the causative agent of the Brazilian zoonosis, named Baggio-Yoshinari Syndrome.

INTRODUCTION

Brazilian spotted fever (BSF), a rickettsial disease caused by *Rickettsia rickettsii*, is the most prevalent tick-borne zoonosis in Brazil, where first reports dated from the 1930s.^{1,2} The Brazilian southeastern states (São Paulo, Minas Gerais, Rio de Janeiro, and Espírito Santo) are considered endemic for BSF, however most of the studies have been conducted in the first three states, with only two reported studies in the state of Espírito Santo.^{3–8}

Lyme disease-like syndrome is an emerging clinical entity that has been described in Brazil, and imitates the clinical manifestations of the classical Lyme disease from the northern hemisphere. However, no spirochetes have been identified in Brazilian patients and ticks, despite exhaustive use of molecular and microbiological assays on clinical samples.⁹ The nomenclature of the Brazilian disease was renamed to Baggio-Yoshinari Syndrome (BYS) during the Rheumatology meeting in São Paulo, 2005, proposing a new tick-borne zoonosis in the country, different from Lyme disease, probably caused by different species of spirochetes or a modified one (Yoshinari NH, unpublished data).

Canine monocytic ehrlichiosis, caused by *Ehrlichia canis*, is the most important tick-borne disease of dogs in Brazil. *Ehrlichia canis* is currently the only ehrlichial species that has been isolated from vertebrates in South America.^{10–12} A preliminary investigation of *Ehrlichia* species was developed in the north and southeastern regions of Brazil, including various tick species, humans, dogs, and capybaras, but no *Ehrlichia* DNA was found in human samples.¹³ In contrast, ehrlichial DNA compatible with *Ehrlichia chaffeensis* and *Ehrlichia ewingii* was recently reported in animal blood samples in Brazil.^{14,15} Human cases of ehrlichiosis in Brazil were only reported by clinical suspects and serological tests, with no DNA amplification and further identification of the agent.^{16,17}

Babesiosis is a common tick-borne disease of dogs and horses in Brazil. *Babesia canis vogeli* (= *B. vogeli*) is the most

common agent affecting dogs, although there are a few reports of *Babesia gibsoni*.^{18–21} Babesiosis in equids are caused mainly by *Babesia caballi* and *Theileria equi* (formerly *Babesia equi*), which are endemic at the tropics, subtropics, and partly in the temperate zones.²² In the North Hemisphere, human babesiosis is found in the United States, where three different species are known to occur, and also in Europe, where the main species identified in humans are *Babesia divergens*, *Babesia microti*, and *Babesia odocolei*.^{23–25} South America has a low number of reports of human babesiosis, including the first suspect case from the State of Pernambuco, a serological study in Colombia, a possible positive case in Poland that seems to have been acquired in Brazil, antibodies to *Babesia* in a study of co-infection with Lyme disease, and a case of a child with hepatoblastoma with a positive blood smear that suggests *Babesia* infection.^{26–30}

With the aim to investigate tick-borne zoonoses and to establish epidemiological data about these diseases in the northern region of the State of Espírito Santo, we visited six counties in this area, and performed serology and molecular biology to the most common tick-borne diseases in humans and domestic animals in Brazil. These counties were selected because of their recent history of BSF laboratory-confirmed cases, including fatal cases (State of Espírito Santo Health Secretary, unpublished data), and/or suspect cases of BYS (Yoshinari NH, unpublished data).

MATERIAL AND METHODS

This study sampled humans, dogs, and horses living in 23 localities in the rural and urban areas within 6 municipalities located in the State of Espírito Santo, Brazil: Nova Venécia, São Mateus, Santa Leopoldina, Ecoporanga, Colatina, and Vila Valério (Figure 1). Samples were collected from May 2007 to March 2008 during three scientific expeditions undertaken to the State of Espírito Santo. In each expedition, farms in rural and homes in urban localities were visited to collect blood samples from humans, dogs, and horses. None of the sampled individuals presented clinical signs of acute infectious diseases when the blood was collected. A total of 109 humans, 85 dogs, and 24 horses were sampled from 22 households (17 farms and

*Address correspondence to Mariana G. Spolidorio, Av. Doutor Arnaldo, 455, sala 3184, Bairro Cerqueira César, São Paulo – SP, CEP 01246-903, Brazil. E-mail: marianaspolidorio@gmail.com

FIGURE 1. Location of six counties in the state of Espírito Santo, southeastern Brazil, where samples were collected for this study.

5 homes). In addition, 92 humans, 7 dogs, and 3 horses were sampled in an Agro-Technical school located in the rural area of Colatina County. Collection of animal and human blood samples were previously approved by Ethical Principles of Ethics Commission to research projects (CAPPesq), Faculty of Medicine, University of São Paulo. Four of the rural households were considered to be the infection source of BSF cases (presumably caused by *R. rickettsii*) confirmed in humans during 2003–2005 with at least 4 fatalities (State of Espírito Santo Health Secretary, unpublished data). The remaining 18 households were considered the probable infection source of Lyme disease-like syndrome (= BYS) among human patients (Yoshinari NH, unpublished data), and were selected because of a large number of *Borrelia burgdorferi*-serologically positive samples sent to the BYS Reference Laboratory at the Faculty of Medicine of the University of São Paulo. Blood samples were collected in two vials: one with EDTA anticoagulant and frozen at -20°C until DNA extraction; and the other without EDTA, from which sera were separated by centrifugation and kept frozen until tested by serological methods.

DNA extraction and polymerase chain reaction. The DNA was extracted from each blood sample using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. DNA samples were eluted in 100 μL of TE buffer. Free DNA water was used as negative control of the extractions. Five microliters of extracted DNA were used for polymerase chain reaction (PCR) amplification. Samples were individually tested by a battery of PCR using the protocols described below.

Primers GE2 (5'-GTT AGT GGC AGA CGG GTG AGT-3') e HE3 (5'-TAT AGG TAC CGT CAT TAT CTT CCC TAT-3') were used to amplify a 360-bp fragment of the 16S ribosomal RNA (rRNA) gene of *Anaplasmataceae*.^{31–33} The PCR were carried out in a total of 50 μL solution containing 1X PCR buffer minus Mg, 1.5 mM MgCl_2 , 0.2 mM dNTPs, 1 U of Platinum TaqDNA Polymerase (Invitrogen, Carlsbad, CA), and 0.2 μM of each primer. The PCR cycle conditions consisted of an initial denaturation for 5 min at 95°C , and 40 repetitive cycles of 15 sec at 95°C , 30 sec at 62°C , and 30 sec at 72°C , followed by a 7 min final extension at 72°C . Positive (previously known blood DNA from an *E. canis*-infected dog) and negative (water) controls were included in all PCR assays.

To amplify a 665-bp fragment of the *Borrelia* flagellin (*flaB*) gene, an initial PCR reaction using primers FLA LL (5'-ACA TAT TCA GAT GCA GAC AGA GGT-3') and FLA RL (5'-GCA ATC ATA GCC ATT GCA GAT TGT-3') were used, and for the nested-PCR that amplifies a 354-bp, primers FLA RS (5'-CTT TGA TCA CTT ATC ATT CTA ATA GC-3') and FLA LS (5'-AAC AGC TGA AGA GCT TGG AAT G-3') were used, with minor modifications.³⁴ The PCR were carried out in a total of 50 μL solution containing 1X PCR buffer minus Mg, 1.5 mM MgCl_2 , 0.2 mM dNTPs, 1 U of Platinum TaqDNA Polymerase, and 0.2 μM of each primer. For all PCR reactions, one positive control (DNA extracted from *Borrelia anserina* culture) and two negative controls (water) were included. Cycling conditions for both reactions included an initial denaturation for 3 min at 95°C , and 40 repetitive cycles of 1 min at 95°C , 1 min at 65°C , and 1 min at 75°C , followed by a 10 min final extension at 75°C .

Polymerase chain reaction to identify *Babesia* spp. was performed using primers BAB-33-57 (5'-GCC AGT AGT CAT ATG CTT GTC TTAA-3') and BAB-432-409 (5'-TTC CTT AGA TGT GGT AGC CGT TTC-3'), corresponding to conserved regions of the 18S rRNA gene of *Babesia* spp., designed to amplify a ≈ 370 -bp portion of the 18S rRNA gene.³⁵ The PCR were carried out in a total of 50 μL solution containing 1X PCR buffer minus Mg, 1.5 mM MgCl_2 , 0.2 mM dNTPs, 1 U of Platinum TaqDNA Polymerase, and 0.2 μM of each primer. Positive (previously known DNA from a *B. canis vogeli*-infected dog) and negative (water) controls were included in all PCR assays. The PCR cycle conditions consisted of an initial denaturation for 3 min at 95°C , and 40 repetitive cycles of 15 sec at 95°C , 30 sec at 63°C , and 30 sec at 72°C , followed by a 7 min final extension at 72°C .

The PCR-positive samples were purified using ExoSap (USB) and sequenced in an automatic sequencer (Applied Biosystems/PerkinElmer, model ABI Prism 310 Genetic, Foster City, CA), according to the manufacturer's protocol. Partial sequences obtained were submitted to BLAST analysis to determine similarities to other species.³⁶

Indirect ELISA to *B. burgdorferi*. Antigen preparation was performed as previously described.³⁷ Indirect ELISA using antigens of *B. burgdorferi* G 39/40, North America origin, was performed by standard method with minor modifications to human samples, and exactly as described when performed to the canine sera samples.^{37,38} Anti-dog immunoglobulin (Ig) G conjugated with alkaline phosphatase produced in rabbit (Sigma-Aldrich, St. Louis, MO) was added in a 1:1000 dilution in blocking solution. Sera of eight dogs from an urban area of São Paulo, without tick-bite history and without visiting rural areas were used as negative controls. As positive control of the reaction, a previously known positive dog serum was used. The same method was applied to the human sera samples, but in this case both IgG and IgM were assayed as previously described.³⁹ Anti-human IgG and Anti-human IgM, both alkaline phosphatase conjugated (Sigma) were diluted 1:1000 in blocking solution. Eight negative human sera samples from people that did not report previous tick-bite and no clinical signs were used as negative controls. As positive control, a known positive human serum to *B. burgdorferi* antigen was used.

Indirect ELISA to horse sera was performed as described, with modifications.⁴⁰ Plates were coated with *B. burgdorferi* antigen in a concentration of 15 $\mu\text{g/mL}$ diluted in carbonate buffer (pH = 9.6). Tween 80 (0.05%) was applied contrary to

Tween 20. Blocking buffer was made with 5% skim milk diluted in phosphate buffered saline (PBS) Tween 80 (pH = 7.4). All serum samples (including eight negative controls) were used in a dilution of 1:400, and positive control was titrated using a 2-fold dilution, starting at 1:400. Anti-horse IgG alkaline phosphatase conjugated (Sigma) was diluted 1:15000 in the PBS Tween 80 buffer, and plates were incubated for 60 min in a wet chamber at room temperature. Thereafter, washing procedures were used and substrate was applied as previously described. Plates were read at 405 nm in a Titertek Multiscan MCC/340 (Flow Laboratories, McLean, VA), and when the first dilution of the positive control serum reached the optical density value near 1.0, the reaction was ready to be read. Cut-off value was established with a confidence degree of 99.9% obtained by considering the mean optical density plus three standard deviations of the eight negative control sera. Optical densities higher than cut-off values were considered positive and the titers were estimated by regression curve.

Immunofluorescence assay (IFA) to *Rickettsia* spp. Immunofluorescence assay slides were prepared using 12-well slides containing crude antigens derived from six *Rickettsia* isolates from Brazil: *R. rickettsii* strain Taitaçu, *R. parkeri* strain At24, *R. bellii* strain Mogi, *R. amblyommii* strain Ac37, *R. rhipicephali* strain HJ#5, and *R. felis* strain Pedreira. Slides were prepared as previously described.⁴¹ Human, canine and horse sera were diluted in 2-fold increments with PBS starting from 1:64 dilution. Ten microliters of the diluted sera were added to each well of the antigen slides. Slides were incubated at 37°C for 30 min in a humid chamber. The slides were rinsed once, and then washed twice for 10 min per wash in PBS. Incubation of the slides was performed with fluorescein isothiocyanate-labeled goat anti-human IgG, goat anti-dog IgG, or goat anti-horse IgG (IgG; Sigma Diagnostics, St. Louis, MO), and washed as described previously. The slides were mounted with buffered glycerin under coverslips. In each slide, a serum previously shown to be nonreactive (negative control) and a known reactive serum (positive control) were tested. For each sample, the endpoint titer reaction with each

of the six rickettsial antigens was determined. Slides were read in an ultraviolet microscope (Olympus BX60, Japan) at 400× magnification.

IFA to *E. canis*. Individual serum samples were tested using *E. canis*-infected DH82 cells as antigen, performed with a Brazilian strain (strain São Paulo) isolated from a naturally infected dog.¹² A fluorescein-conjugated anti-dog IgG (Sigma) previously titrated to the best working dilution (1:600) was used as described.^{12,42} To the human sera, anti-human IgG fluorescein-conjugated (Sigma) was used in the 1:400 dilution. Serum was considered to contain antibodies reactive to *E. canis* if it displayed a reaction at the 1:64 dilution. Blocking solution (bovine serum albumin [BSA] 1% in PBS for canine sera, and inactivated rabbit sera 2% in PBS for human sera) were used to diminish background. In each slide, a serum previously shown to be nonreactive (negative control) and a known reactive serum (positive control) were tested. Samples that reacted at the screening dilution (1:64) were titrated using serial 2-fold dilutions to determine endpoint titers.

IFA to *Babesia* spp. and *Theileria equi*. Antigens of *B. canis vogeli*, *Babesia bovis*, and *T. equi* were prepared as described.⁴³ Briefly, serum dilution tested against *B. canis vogeli* was 1:40, and to *B. bovis* and *T. equi* was 1:80. After washing with PBS buffer, slides were incubated with the corresponding fluorescein-conjugate. Anti-horse IgG FITC (Sigma) was diluted 1:32; anti-dog IgG FITC (Sigma) was diluted 1:200; and anti-human IgG FITC (Sigma) was diluted 1:400.⁴⁴

RESULTS AND DISCUSSION

Results of all serological tests and PCR performed on human, dog, and horse blood samples are shown in Table 1. Data were separated according to rural and urban areas. In addition, results for the Agro-Technical school were presented separately because half of the human samples were collected from this single site.

Human samples were all negative for *Babesia*, both by serology and PCR. A few human or horse sera were reactive

TABLE 1

Results of serological tests and polymerase chain reaction (PCR) performed on blood samples of humans, dogs, and horses from six municipalities in the state of Espírito Santo, southeastern Brazil*

Agents investigated	No. individuals that showed to be positive (%)										
	Humans				Dogs				Horses		
	Rural (N = 100)	Urban (N = 9)	School (N = 92)	Total (N = 201)	Rural (N = 71)	Urban (N = 14)	School (N = 7)	Total (N = 92)	Rural (N = 24)	School (N = 3)	Total (N = 27)
Serology											
<i>Borrelia burgdorferi</i>	8 (8.0)	1 (11.1)	0 (0)	9 (4.5)	38 (53.5)	7 (50.0)	2 (28.6)	47 (51.1)	1 (4.2)	0 (0)	0 (0)
<i>Rickettsia rickettsii</i>	31 (31.0)	2 (22.2)	16 (17.4)	49 (24.3)	4 (5.6)	0 (0)	0 (0)	4 (4.3)	4 (16.7)	3 (100)	7 (25.9)
<i>R. parkeri</i>	28 (28.0)	2 (22.2)	21 (22.8)	41 (20.4)	2 (2.8)	0 (0)	0 (0)	2 (2.2)	2 (8.3)	2 (66.7)	4 (14.8)
<i>R. amblyommii</i>	33 (33.3)	3 (33.3)	16 (17.4)	52 (25.9)	3 (4.2)	0 (0)	0 (0)	3 (3.3)	3 (2.5)	2 (66.7)	5 (18.5)
<i>R. rhipicephali</i>	36 (36.0)	4 (44.4)	17 (18.5)	57 (28.3)	2 (2.8)	1 (7.1)	0 (0)	3 (3.3)	3 (2.5)	2 (66.7)	5 (18.5)
<i>R. felis</i>	31 (31.0)	4 (44.4)	16 (17.5)	51 (25.3)	2 (2.8)	2 (14.3)	0 (0)	4 (4.3)	1 (4.2)	2 (66.7)	4 (14.8)
<i>R. bellii</i>	17 (17.0)	2 (22.2)	10 (10.9)	39 (19.4)	3 (4.2)	1 (7.1)	0 (0)	4 (4.3)	2 (8.3)	3 (100)	5 (18.5)
<i>Ehrlichia canis</i>	3 (3.0)	0 (0)	1 (1.1)	4 (2.0)	33 (46.5)	4 (28.6)	1 (14.3)	38 (41.3)	ND	ND	–
<i>Babesia canis vogeli</i>	0 (0)	0 (0)	0 (0)	0 (0)	25 (46.5)	6 (42.9)	0 (0)	31 (33.7)	ND	ND	–
<i>Babesia bovis</i>	0 (0)	0 (0)	0 (0)	0 (0)	ND	ND	ND	–	ND	ND	–
<i>Theileria equi</i>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	24 (100)	3 (100)	27 (100)
PCR											
<i>Anaplasmatidae</i>	0 (0)	0 (0)	0 (0)	0 (0)	22 (31.0)	3 (21.4)	0 (0)	25 (27.2)	0 (0)	0 (0)	0 (0)
<i>Borrelia</i>	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>Babesia/Theileria</i>	0 (0)	0 (0)	0 (0)	0 (0)	14 (19.7)	6 (42.9)	0 (0)	20 (21.7)	6 (25.0)	0 (0)	6 (22.2)
<i>Hepatozoon</i> †	ND	ND	ND	–	46 (64.8)	8 (57.1)	0 (0)	54 (58.7)	ND	ND	–

*ND = not determined.

†Previously reported data.⁴⁵

for *B. burgdorferi* by serology. On the other hand, approximately half of the dogs were serologically reactive to *B. burgdorferi*. To exclude the possibility of a cross-reaction between *Borrelia* spp. and *Leptospira* spp., these canine serum samples were also tested by serology using 23 different serovars of *Leptospira* sp. (data not shown), but there was low reactivity to *Leptospira* sp. (14%), and no significance correlation within the results (P value = 0.568) (data not shown). Differing from the United States, Europe, and Asia, no *Ixodes ricinus*-complex ticks has been reported in Brazilian BYS-endemic areas. Maximum titers achieved to *B. burgdorferi* in human samples were 1:800 for IgG and 1:200 for IgM. In Brazil, *B. burgdorferi* sensu lato spirochetes have never been isolated from humans or ticks. In addition, serology to *B. burgdorferi* antigens usually presents low titers that decrease to disappearance fast. We have also tested the human sera of this study with *B. burgdorferi* sensu stricto antigens by western blot, resulting in no reaction compatible with Lyme disease (data not shown). For these reasons, a new syndrome has been described in the country.⁴⁵ Interestingly, a relapsing fever-like spirochete, namely *Borrelia brasiliensis*, has been isolated in southern Brazil from *Ornithodoros brasiliensis*, a soft tick locally known as the “dog tick.”⁴⁶ It is possible that soft ticks, yet to be documented in the areas of this study, have vectored relapsing fever spirochetes to dogs, contributing to the canine spirochetal reactivity.

Overall, sera from 70 (34.8%) humans, 7 (7.6%) dogs, and 7 (25.9%) horses were reactive to at least one *Rickettsia* species. Endpoint titers of human sera varied from 64 to 1,024 to all six *Rickettsia* species. Endpoint titers of dog sera varied from 64 to 32,768 for *R. rickettsii*, 256 to 16,384 for *R. parkeri*, 64 to 4,096 for *R. amblyommii*, *R. rhipicephali*, *R. bellii*, and *R. felis*. Endpoint titers of horse sera varied from 64 to 512 for *R. rickettsii*, 64 to 128 for *R. parkeri* and *R. felis*, 128 to 1,024 for *R. amblyommii*, and 128 to 512 to *R. rhipicephali* and *R. bellii*. Considering individual sera, in all cases endpoint titers for two or more *Rickettsia* species were less than 4-fold different, except for one case, a dog from a household in the rural area of Santa Leopoldina municipality, which presented endpoint titer of 1,024 to *R. felis*, 128 to *R. bellii*, and non-reactive for the remaining *Rickettsia* species. Because of this greater difference between titers, the serological response in this dog was considered to be elicited by *R. felis*. In fact, *R. felis*-infected fleas (*Ctenocephalides felis felis*) collected on dogs were also observed in both urban and rural areas of this study (Labruna and others, unpublished data). These results suggest that part of the serologically reactive human sera of the present study could indicate past infection by *R. felis*.

Our serological results, coupled with molecular assays, indicate that a significant amount of dogs were infected by *E. canis*. Although no human blood showed to contain ehrlichial DNA, at least 2% of the human sera were reactive to *E. canis* antigen by IFA. Because *E. canis* has been shown to infect humans in Venezuela, these serological responses to *E. canis* in this study could represent accidental infection by *E. canis*, since *R. sanguineus* was a common finding on dogs of the households of both urban and rural areas of the present study.^{35,47}

For most of the agents investigated, similar results were obtained for urban and rural humans and dogs. One could find these results unpredictable because urban and rural areas have lots of ecological differences, although in all selected municipalities, separation of the urban and rural environments is very tenuous. However, all urban households of this study

were selected because of recent history of Lyme Disease-like Syndrome among residents. In this case, they all claimed about previous tick infestations when visiting rural areas (data not shown).

Both human and horse samples from the Agro-Technical School showed serological positive reactions to the six *Rickettsia* species, in contrast to canine samples that showed no reaction. In 2005, the death of two humans from the school was assumed to be caused by a spotted fever group (SFG) *Rickettsia* (State of Espírito Santo Health Secretary, unpublished data). The majority of people from the school, sampled for this study, reported frequent tick bites within the school area. Part of these ticks were collected by us and identified as *Amblyomma cajennense* and *Amblyomma dubitatum* (data not shown). Possibly, the few dogs sampled in the school are less exposed to these ticks, because they were housed close to the main buildings, far from the tick-infested fields where the students regularly had field classes and horses were used during work.

Assembling our results, we can infer that the north part of the State of Espírito Santo can be considered an endemic area for SFG *Rickettsia* but no former considerations can be made about the other organisms regarding human population, although in domestic animals it was possible to conclude that *E. canis* and *Babesia* spp. were circulating while we surveyed these places, contributing to the conclusion that these areas are endemic to some other tick-borne diseases in animals. *Hepatozoon canis* was also found infecting dogs, as previously described.³⁵ Further studies in other parts of Brazil, focusing attention on tick species that bites humans, would contribute for a better elucidation on tick-borne zoonoses.

Received October 6, 2009. Accepted for publication March 26, 2010.

Acknowledgments: We are grateful to Melanie Gutjahr (Laboratory of Epidemiology of the Faculty of Veterinary Medicine of the University of São Paulo) for preparing Figure 1 of the present study, Denise M. Mancini [Coordenação Geral de Laboratórios de Saúde Pública (CGLAB), Secretaria de Vigilância em Saúde (SVS) do Ministério da Saúde (MS)], and Regina M. B. Terrão (Lacen Espírito Santo) for logistic support in Espírito Santo, and to the “Agentes Locais de Vigilância Ambiental em Saúde do Departamento de Vigilância Ambiental do Município de Nova Venécia, ES” for their valuable help during field work.

Financial support: This work was supported by the Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP grant 07/51899-2 to N.H.Y.), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES PhD scholarship to M.G.S.), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq career scholarship to M.B.L.).

Authors' addresses: Mariana G. Spolidorio and Natalino H. Yoshinari, Laboratório de Investigação Médica 17 (LIM17), Faculdade de Medicina, Universidade de São Paulo, São Paulo, Brazil, E-mails: marianaspolidorio@gmail.com and yoshinari@lim17.fm.usp.br. Marcelo B. Labruna, Jonas Moraes-Filho, Iara Silveira, and Sônia R. Pinheiro, Departamento de Medicina Veterinária Preventiva e Saúde Animal, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Cidade Universitária, São Paulo, Brazil, E-mails: labruna@usp.br, jonasmfilho@hotmail.com, iarasily@yahoo.com.br, and soniapi@usp.br. Rosângela Z. Machado, Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista “Julio de Mesquita Filho,” Jaboticabal, Brazil, E-mail: zacarias@fcav.unesp.br. Augusto M. Zago and Késia M. Caliani, Núcleo de Vigilância em Saúde, Superintendência Regional de Saúde de Colatina, Colatina, Brazil, E-mails: augustozago@saude.es.gov.br and kesiamargotto@saude.es.gov.br. Dirlei M. Donatele, Centro Universitário São Camilo Espírito Santo – CUSC, Cachoeiro de Itapemirim, Brazil.

REFERENCES

- Dias E, Martins AV, 1939. Spotted fever in Brazil. A summary. *Am J Trop Med* 19: 103–108.
- Piza JT, Meyer JR, Gomes LS, 1993. *Typho Exantematico de São Paulo*. São Paulo, Brazil: Sociedade Imprensa Paulista.
- Galvão MA, Silva LJ, Nascimento EM, Calic SB, Sousa R, Bacellar F, 2005. Riquetsioses no Brasil e Portugal: ocorrência, distribuição e diagnóstico. *Rev Saude Publica* 39: 850–856.
- Angerami RN, Resende MR, Feltrin AF, Katz G, Nascimento EM, Stucchi RS, Silva LJ, 2006. Brazilian spotted fever: a case series from an endemic area in southeastern Brazil: epidemiological aspects. *Ann NY Acad Sci* 1078: 170–172.
- Lamas C, Favacho A, Rozenal T, Bóia MN, Kirsten AH, Guterres A, Barreira J, de Lemos ER, 2008. Characterization of *Rickettsia rickettsii* in a case of fatal Brazilian spotted fever in the city of Rio de Janeiro, Brazil. *Braz J Infect Dis* 12: 149–151.
- Labruna MB, 2009. Ecology of *Rickettsia* in South America. *Ann NY Acad Sci* 1166: 156–166.
- Sexton DJ, Muniz M, Corey GR, Breitschwerdt EB, Hegarty BC, Dumler S, Walker DH, Pecanha PM, Dietze R, 1993. Brazilian spotted fever in Espírito Santo, Brazil: description of a focus of infection in a new endemic region. *Am J Trop Med Hyg* 49: 222–226.
- Oliveira KA, Oliveira LS, Dias CC, Silva A Jr, Almeida MR, Almada G, Bouyer DH, Galvão MA, Mafra C, 2008. Molecular identification of *Rickettsia felis* in ticks and fleas from an endemic area for Brazilian Spotted Fever. *Mem Inst Oswaldo Cruz* 103: 191–194.
- Mantovani E, Costa IP, Gauditano G, Bonoldi VL, Higuchi ML, Yoshinari NH, 2007. Description of lyme disease-like syndrome in Brazil. Is it a new tick borne disease or lyme disease variation? *Braz J Med Biol Res* 40: 443–456.
- Dumler JS, Barbet AF, Bekker CP, Dasch GA, Palmer GH, Ray SC, Rikihisa Y, Rurangirwa FR, 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and “HGE agent” as subjective synonyms of *Ehrlichia phagocytophila*. *Int J Syst Evol Microbiol* 51: 2145–2165.
- Torres HM, Massard CL, Figueiredo MJ, Ferreira T, Almosny NRP, 2002. Isolamento e propagação da *Ehrlichia canis* em células DH82 e obtenção de antígeno para a reação de imunofluorescência indireta. *Rev Bras Ciênc Vet* 9: 77–82.
- Aguiar DM, Saito TB, Hagiwara MK, Machado RZ, Labruna MB, 2007. Serological diagnosis of canine monocytic ehrlichiosis with Brazilian antigen of *Ehrlichia canis*. *Cienc. Rural* 37: 796–802.
- Labruna MB, McBride JW, Camargo LM, Aguiar DM, Yabsley MJ, Davidson WR, Stromdahl EY, Williamson PC, Stich RW, Long SW, Camargo EP, Walker DH, 2007. A preliminary investigation of *Ehrlichia* species in ticks, humans, dogs, and capybaras from Brazil. *Vet Parasitol* 143: 189–195.
- Machado RZ, Duarte JM, Dagnone AS, Szabó MP, 2006. Detection of *Ehrlichia chaffeensis* in Brazilian marsh deer (*Blastocerus dichotomus*). *Vet Parasitol* 139: 262–266.
- Oliveira LS, Oliveira KA, Mourão LC, Pescatore AM, Almeida MR, Conceição LG, Galvão MA, Mafra C, 2009. First report of *Ehrlichia ewingii* detected by molecular investigation in dogs from Brazil. *Clin Microbiol Infect* 15: 55–56.
- Calic SB, Galvão MA, Bacellar F, Rocha CM, Mafra CL, Leite RC, Walker DH, 2004. Human ehrlichioses in Brazil: first suspect cases. *Braz J Infect Dis* 8: 259–262.
- Costa PS, Valle LM, Brigatte ME, Greco DB, 2006. More about human monocytotropic ehrlichiosis in Brazil: serological evidence of nine new cases. *Braz J Infect Dis* 10: 7–10.
- Passos LM, Geiger SM, Ribeiro MF, Pfister K, Zahler-Rinder M, 2005. First molecular detection of *Babesia vogeli* in dogs from Brazil. *Vet Parasitol* 127: 81–85.
- Trapp SM, Messick JB, Vidotto O, Jojima FS, de Moraes HS, 2006. *Babesia gibsoni* genotype Asia in dogs from Brazil. *Vet Parasitol* 141: 177–180.
- Costa-Júnior LM, Ribeiro MF, Rembeck K, Rabelo EM, Zahler-Rinder M, Hirzmann J, Pfister K, Passos LM, 2009. Canine babesiosis caused by *Babesia canis vogeli* in rural areas of the State of Minas Gerais, Brazil and factors associated with its seroprevalence. *Res Vet Sci* 86: 257–260.
- O'Dwyer LH, Lopes VV, Rubini AS, Paduan Kdos S, Ribolla PE, 2009. *Babesia* spp. infection in dogs from rural areas of São Paulo State, Brazil. *Rev Bras Parasitol Vet* 18: 23–26.
- Friedhoff KT, 1982. Piroplasmas of horses—impact on the international horse trade. *Berl Munch Tierarztl Wochenschr* 95: 368–374.
- Hildebrandt A, Hunfeld KP, Baier M, Krumbholz A, Sachse S, Lorenzen T, Kiehntopf M, Fricke HJ, Straube E, 2007. First confirmed autochthonous case of human *Babesia microti* infection in Europe. *Eur J Clin Microbiol Infect Dis* 26: 595–601.
- Hunfeld KP, Hildebrandt A, Gray JS, 2008. Babesiosis: recent insights into an ancient disease. *Int J Parasitol* 38: 1219–1237.
- Herwaldt BL, Cacciò S, Gherlinzoni F, Aspöck H, Slemenda SB, Piccaluga P, Martinelli G, Edelhofer R, Hollenstein U, Poletti G, Pampiglione S, Löschenberger K, Tura S, Pieniazek NJ, 2003. Molecular characterization of a non-*Babesia divergens* organism causing zoonotic babesiosis in Europe. *Emerg Infect Dis* 9: 942–948.
- Alecrist I, Pinto B, Avila T, Costa R, Pessoa I, 1983. Registro do primeiro caso de infecção humana por *Babesia* spp. no Brasil. *Rev Patol Trop* 12: 11–29.
- Rios L, Alvarez G, Blair S, 2003. Serological and parasitological study and report of the first case of human babesiosis in Colombia. *Rev Soc Bras Med Trop* 36: 493–498.
- Humiczewska M, Kuźna-Grygiel W, 1997. A case of imported human babesiosis in Poland. *Wiad Parazytol* 43: 227–229.
- Yoshinari NH, Abrão MG, Bonoldi VL, Soares CO, Madruga CR, Scofield A, Massard CL, da Fonseca AH, 2003. Coexistence of antibodies to tick-borne agents of babesiosis and lyme borreliosis in patients from Cotia county, State of São Paulo, Brazil. *Mem Inst Oswaldo Cruz* 98: 311–318.
- Rech A, Bittar CM, de Castro CG, Azevedo KR, dos Santos RP, Machado AR, Schwartzmann G, Goldani L, Brunetto AL, 2004. Asymptomatic babesiosis in a child with hepatoblastoma. *J Pediatr Hematol Oncol* 26: 213.
- Anderson BE, Sumner JW, Dawson JE, Tzianabos T, Greene CR, Olson JG, Fishbein DB, Olsen-Rasmussen M, Holloway BP, George EH, 1992. Detection of the etiologic agent of human ehrlichiosis by polymerase chain reaction. *J Clin Microbiol* 30: 775–780.
- Breitschwerdt EB, Hegarty BC, Hancock SI, 1998. Sequential evaluation of dogs naturally infected with *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia equi*, *Ehrlichia ewingii*, or *Bartonella vinsonii*. *J Clin Microbiol* 36: 2645–2651.
- Aguiar DM, Hagiwara MK, Labruna MB, 2008. *In vitro* isolation and molecular characterization of an *Ehrlichia canis* strain from São Paulo, Brazil. *Braz J Microbiol* 39: 489–493.
- Stromdahl EY, Williamson PC, Kollars TM Jr, Evans SR, Barry RK, Vince MA, Dobbs NA, 2003. Evidence of *Borrelia lonestari* DNA in *Amblyomma americanum* (Acari: Ixodidae) removed from humans. *J Clin Microbiol* 41: 5557–5562.
- Spolidorio MG, Labruna MB, Zago AM, Donatelle DM, Caliri KM, Yoshinari NH, 2009. *Hepatozoon canis* infecting dogs in the state of Espírito Santo, southeastern Brazil. *Vet Parasitol* 163: 357–361.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ, 1990. Basic local alignment search tool. *J Mol Biol* 215: 403–410.
- Joppert AM, Hagiwara MK, Yoshinari NH, 2001. Antibodies in dogs from Cotia county, São Paulo State, Brazil. *Revta Inst Med Tropical* 43: 251–255.
- Voller A, Bidwell DE, Bartlett A, 1976. Enzyme immunoassays in diagnostic medicine. Theory and practice. *Bull World Health Organ* 53: 55–65.
- Naka EN, Costa IP, Araújo CA, Soares CO, Yoshinari NH, 2008. Pesquisa de anticorpos anti-*Borrelia* e anti-*Babesia* em soro de crianças com manifestações clínicas e epidemiologia compatíveis com a doença de lyme-simile no estado de mato grosso do sul. *Rev Bras Reumatol* 48: 74–85.
- Salles RS, Fonseca AH, Scofield A, Madureira RC, Yoshinari NH, 2002. Sorologia para *Borrelia burgdorferi* latu sensu em equinos no estado do Rio de Janeiro. *Hora Vet* 127: 46–49.
- Labruna MB, Horta MC, Aguiar DM, Cavalcante GT, Pinter A, Gennari SM, Camargo LM, 2007. Prevalence of *Rickettsia* infection in dogs from the urban and rural areas of Monte Negro

- municipality, western Amazon, Brazil. *Vector Borne Zoonotic Dis* 7: 249–255.
42. Ristic M, Huxsoll DL, Weisiger RM, Hildebrandt PK, Nyindo MB, 1972. Serological diagnosis of tropical canine pancytopenia by indirect immunofluorescence. *Infect Immun* 6: 226–231.
 43. Baldani CD, Machado RZ, Raso TF, Pinto AA, 2007. Serodiagnosis of *Babesia equi* in horses submitted to exercise stress. *Pesqui Vet Bras* 27: 179–183.
 44. Callow LL, McGregor W, Rodwell BJ, Rogers RJ, Fraser GC, Mahoney DF, Robertson GM, 1979. Evaluation of an indirect fluorescent antibody test to diagnose *Babesia equi* infection in horses. *Aust Vet J* 55: 555–559.
 45. Shinjo SK, Gauditano G, Marchiori PE, Bonoldi VL, Costa IP, Mantovani E, Yoshinari NH, 2009. Manifestação neurológica na síndrome de Baggio-Yoshinari (síndrome brasileira semelhante à doença de lyme). *Revista Brasileira de Reumatologia* 49: 492–498.
 46. Davis GE, 1952. Observations on the biology of the argasid tick, *Ornithodoros brasiliensis* Aragão, 1923, with the recovery of a spirochete, *Borrelia brasiliensis*, n. sp. *J Parasitol* 38: 473–476.
 47. Perez M, Bodor M, Zhang C, Xiong Q, Rikihisa Y, 2006. Human infection with *Ehrlichia canis* accompanied by clinical signs in Venezuela. *Ann N Y Acad Sci* 1078: 110–117.