Rev Soc Bras Med Trop 49(5):567-571, September-October, 2016
doi:10.1590/0037-8682-0023-2016

Major Article

Antibodies for *Rickettsia* spp. in patients with negative serology for dengue virus, leptospirosis, and meningococcal disease in municipalities of São Paulo State, Brazil

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Abstract

Introduction: Brazilian spotted fever is an infectious disease with a high mortality rate if not treated early. Differential diagnosis is difficult, as the first clinical signs are non-specific and can be confused with other diseases. The aim of the study was to investigate evidence of infection with *Rickettsia rickettsii* and *Rickettsia parkeri* in negative sera samples, collected in 2014, from patients with suspected leptospirosis, dengue fever, and meningococcal disease in Atibaia and Bragança Paulista municipalities of the State of São Paulo. **Methods**: The samples stored at the Institute Adolfo Lutz in Campinas were tested using an indirect immunofluorescence assay (IFA) with IgG and IgM against *R. rickettsii* and *R. parkeri*. Real-time polymerase chain reaction (PCR) testing was performed for the sera samples of patients who died (n = 3), those with initial suspicion of meningococcal disease (n = 6), and those with positive IFA results. **Results**: Of 258 samples from Bragança Paulista, 4 (1.6%) were positive, with IgG titers of 1:64 and 1:128 against *R. rickettsii* and *R. parkeri*, respectively. Of 155 samples from Atibaia, 2 (1.3%) were positive, with IgG titers of 1:64 and 1:128 against *R. rickettsii* and *R. parkeri*, respectively. No sample showed positive PCR results. **Conclusions**: This serological investigation suggests there is evidence of exposure to *Rickettsia* spp. in residents of areas that have environmental conditions favorable to the spread of bacteria, in which Brazilian spotted fever incidence was not previously confirmed.

Keywords: Brazilian spotted fever. *Rickettsia rickettsii*. *Rickettsia parkeri*. Indirect immunofluorescence test. Differential diagnosis.

INTRODUCTION

Brazilian spotted fever (BSF) is an infectious disease, marked by acute fever, with a high mortality rate when not diagnosed and treated early. In Brazil, it is considered a re-emerging zoonosis of great importance to public health because of the difficulty in establishing a clinical diagnosis, the high rate of mortality, and the involvement of an economically active population[1].

Brazilian spotted fever is caused by *Rickettsia rickettsii*, a Gram-negative pleomorphic bacterium[2], and transmitted in Brazil by ticks of the genus *Amblyomma*, notably *Amblyomma sculptum* and *Amblyomma aureolatum*[3] [4]. *Rickettsia parkeri* also belongs to *Rickettsia* spp. that cause BSF and has been associated with milder forms of the disease[5] [6].

According to data from the Brazilian epidemiological surveillance system, between 2000 and June 2015, 1,387 cases of BSF were recorded in the country, with 417 deaths, representing a lethality rate of 30%. Of the 1,387 total cases, 1,009 (72.7%) were confirmed in the Southeast region, where 414 (99.3%) of a total of 417 BSF-related deaths were recorded.

Both domestic and wild animals play an important role as vertebrate hosts in the epidemiology of BSF by temporarily developing rickettsemia (over the course of days or weeks), favoring the infection of new ticks by *R. rickettsii*. Hosts are also important as they transport vectors to environments in and around places where humans live. They are called amplifier hosts and include animals such as the capybara (*Hydrochoerus hydrochaeris*), opossum (*Didelphis* spp.), and domestic dog (*Canis familiaris*)[7] [8] [9]. Horses and dogs with positive serology are often found in endemic regions. These animals have been

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Received 9 June 2016
Accepted 14 September 2016

considered sentinels and are important in epidemiological studies of BSF, as positive serology is related to the circulation of the bacteria, particularly if the animals are autochthonous[10][11].

In the State of São Paulo, Campinas is the region with the highest number of cases from 1998 to 2012 (294, or 53% of the total number of cases in the country), followed by metropolitan São Paulo (98, or 17.5%), the region of Piracicaba (59, or 10.6%), and the coastal region (28, or 5%). According to the Epidemiological Surveillance Center, most of the confirmed cases occurred in municipalities belonging to the basins of the Piracicaba, Capivari, and Jundiaí (PCJ) rivers.

Atibaia and Bragança Paulista are located in the PCJ Basin in the Northeast region of the State of São Paulo and had no reports of confirmed human cases of BSF in the last 25 years, even though there is evidence of *R. rickettsii* circulation among horses and the presence of vectors and capybaras in these areas[12].

In endemic areas, BSF in the initial clinical phase is part of the differential diagnosis of acute febrile exanthematic syndromes. Due to non-specific symptoms, the diagnosis is difficult, particularly in the early days of the disease[5]. Thus, given the similarity of the clinical picture with endemic diseases such as leptospirosis, meningococcal disease, and dengue fever, the present study aimed to investigate evidence of infection by *Rickettsia* spp. (*R. rickettsii* and *R. parkeri*) in patients with negative serology and no confirmation for such diseases in residents of Atibaia and Bragança Paulista.

METHODS

This is a descriptive study of the prevalence of antibodies against *R. rickettsii* and *R. parkeri* in negative serum samples for dengue virus (DV), leptospirosis, and meningococcal disease, stored in a public health reference laboratory, Adolfo Lutz Institute in Campinas, State of São Paulo.

The inclusion criteria of the samples were as follows: collected 1) from residents of the municipalities of Atibaia and Bragança Paulista, in 2014 and in the period potentially associated with the increase of antibodies after the first symptoms, and 2) from patients with non-confirmed initial suspicion of dengue fever and/or leptospirosis and meningococcal disease.

Bragança Paulista and Atibaia were chosen because they are considered non-endemic for BSF, because there is evidence of *R. rickettsii* transmission in horses, and because they are located in the PCJ Basin, the most endemic region of BSF in the State of São Paulo[12].

The samples were tested by indirect immunofluorescence assay (IFA), taking into consideration that the period in which antibody titers increased varied approximately between 7 and 10 days after the onset of BSF symptoms. However, the only samples processed were those in which the period between the onset of symptoms and sample collection was equal or superior to 6 days.

For Atibaia, we identified 155 samples that could be subjected to analysis by IFA, of which 10 were negative for leptospirosis, 143 for dengue virus, and 2 for meningococcal disease in counter-immunoelectrophoresis. For Bragança Paulista, we identified 258 samples, of which 20 were negative for leptospirosis, 234 for dengue virus, and 4 for meningococcal disease in counter-immunoelectrophoresis.

Indirect immunofluorescence assay was conducted by using 12 hole blades sensitized with *R. rickettsii* and *R. parkeri* antigens and stored at -20° C. Sera were diluted in phosphate-buffered saline, starting from a 1:64 dilution (315µL of phosphate-buffered saline and 5µL of serum). A positive and a negative control were included in each slide. The slides were incubated in humid chambers in bacteriological incubators at 37°C for 30 minutes. The slides were then washed with a buffer and distilled water and left to dry naturally.

Conjugates of human immunoglobulin G (IgG) antibody, diluted to a ratio of 1:80 (15µL of conjugate in 1,185µL of Evans blue) were then used to perform the indirect IFA as described by Moares Filho et al.[13]. Sera that reacted to the 1:64 dilution were titrated to a dilution of 1:512. A real-time polymerase chain reaction (PCR) was performed for samples from patients that died (n = 3) and those with initial suspicion of meningococcal disease (n = 6), since qPCR is primarily indicated for severe cases.

Immunofluorescence-positive samples (IgG) from the 1:64 dilution were also processed for titration of immunoglobulin M (IgM) with the use of PCR. The clinical symptoms of positive patients were obtained from reporting forms of epidemiological surveillance databases of the municipalities.

Ethicals considerations

This project was approved by the Research Ethics Committee of the Faculdade de Ciências Médicas under the process reference: CAAE 35540114.3.0000.5404 and by the Ethics Committee of the Institute Adolfo Lutz of São Paulo under the process reference: CAAE 35540114.3.3001.0059.

RESULTS

A total of 413 samples were taken from patients from the municipalities of Atibaia (n = 155) and Bragança Paulista (n = 258). Using IFA, we obtained six positive samples, 1.3% (2/155) in Atibaia and 1.6% (4/258) in Bragança Paulista (**Table 1**).

Among the reactive samples in Atibaia, most initial suspicions were for dengue fever (5/6), with one suspected case of leptospirosis. The average age of the patients was 34.3 years, including four men and two women. The collection period ranged from 6 to 13 days after the onset of symptoms.

Samples from deceased patients (n = 3) and those with early suspicion of meningococcal disease (n = 6) returned negative results using qPCR. The six positive IFA samples were also processed by PCR and tested negative (**Table 1**).

Samples with positive IFA results were collected at different times of the year: March, April, May, June, September, and November. Most (4, or 80%), were in the first semester in both municipalities, a period in which the highest occurrence of dengue feverand leptospirosis were observed.

Signs and symptoms presented by patients whoseserum was reactive to IFA are described in **Table 2**. We did not

TABLE 1

Epidemiological history and IgM and IgG titers against *Rickettsia rickettsii* and *Rickettsia parkeri*, and polymerase chain reaction results in patients with immunofluorescence assay-positive samples from Atibaia and Bragança Paulista, State of São Paulo, 2014.

Patient	Contact with ticks	Contact with dogs with ticks	Visit to the countryside	Titers against *R. rickettsii* IFA		Titers against *R. parkeri* IFA		PCR
				IgM	IgG	IgM	IgG	
1	No	No	No	1:64	1:128	1:64	<1:64	Negative
2	No	No	No	1:128	1:64	1:64	<1:64	Negative
3	No	Yes	No	<1:64	1:64	<1:64	<1:64	Negative
4	No	No	Yes	<1:64	1:64	1:64	<1:64	Negative
5	Yes	Yes	No	<1:64	1:128	<1:64	1:128	Negative
6	No	No	No	<1:64	<1:64	<1:64	1:64	Negative

R.: *Rickettsia;* **IgM:** immunoglobulin M; **IgG:** immunoglobulin G; **IFA**: immunofluorescence assay; **PCR:** polymerase chain reaction.

TABLE 2

Percent distribution of signs and symptoms of patients with IFA-positive samples (n = 6) in the municipalities of Atibaia and Bragança Paulista, State of São Paulo, 2014.

Signs and symptoms	Number	Percentage
Fever	5	83.0
Headache	4	67.0
Arthralgia	3	50.0
Myalgia	3	50.0
Prostation	2	33.0
Jaundice	1	17.0

IFA: immunofluorescence assay; **n:** number

detect the appearance of petechiae, skin rash, or hemorrhagic manifestations in any of the six patients. Patient 6 reported antibiotic usage (penicillin) during symptoms.Patients 3 and 5 reported contact with dogs with ticks during the illness period, and patient 4 had been in the countryside (**Table 1**).

DISCUSSION

The seroprevalence (IFA) for *Rickettsia* spp. in negative samples for dengue virus, leptospirosis, and meningococcal disease in Atibaia and Bragança Paulista were 1.3% and 1.6%, respectively. Six samples titrated against *R. rickettsii* and *R. parkeri* showed detectable titers (≥ 1:64) and were considered positive for rickettsial infections. We identified two patients in Atibaia with titers of IgM and IgG reactive against *R. rickettsii*, one presenting an IgG titer of 1:128 and the other an IgM titer of 1:128. In a patient from Bragança Paulista, we observed IgG titers of 1:128 (*R. rickettsii* and *R. parkeri*), suggesting exposure to and possible infection with *Rickettsia*. However, when considering that the clinical picture resulted from an infection by *Rickettsia* spp., the occurrence of titers considered borderline (1:64 in patients 3, 4, and 6) can be explained by the early collection.

We chose to test for more than one antigen owing to the suspicion of a possible association of *R. parkeri* to milder clinical cases, similar to what has been observed in the States of Santa Catarina[5] and São Paulo[6]. In addition, we found individuals with prior contact with *Rickettsia*, although we were unable to confirm whether the contact occurred in the PCJ Basin or elsewhere. Previously published data suggest circulationof *Rickettsia*in other municipalities in the same area[14] [15].Cross-reaction between *Rickettsia* spp. is possible, especially among *R. rickettsii* and *R. parkeri*; this may frequently hinder accurate identification of the etiologic agent in an infection[16]. Thus, serological test using only *R. rickettsii* antigen can generate not only false negative but also false positive results of infection caused by other bacteria of spotted fever group, thus limiting the clinical diagnosis and understanding of *Rickettsia* distribution. In a serological study of patients presenting with spotted fever in North Carolina, USA, cases of infection with *R. parkeri* and *R. amblyommii* were identified in patients with an initial suspicion of *R. rickettsii*[17].

While the observed titers in patients are not necessarily associated with the clinical picture (in general, *flu-like* or *dengue-like*), in three patients (3, 4, and 5) there was an epidemiological history that could explain possible exposure to the vector and subsequent *Rickettsia* infection.

Contact with dogs parasitized by *A. aureolatum* or *Rhipicephalus sanguineus* and environments where these dogs present are potentially relevant epidemiological factors to BSF transmission, since these species of ticks may also parasitize humans[18]. *Amblyomma aureolatum* is considered the main vector of *R. rickettsii* in the São Paulo region[19] and has been reported to frequently parasitize dogs in counties (Nazaré Paulista and Mairiporã) close to the study region[20]. Moreover, based on the molecular characterization of *R. rickettsii* in *R. sanguineus* in Brazil, the possible risk of an active, or even an indirect, participation of *R. sanguineus*in the transmission

of *R. rickettsii* to humans cannot be excluded[21]. In addition, a potential risk of transmission of BSF or other rickettsial infectionsviaparasitized dogs cannot be ruled out. In this respect, there is a recent report regarding a Rocky Mountain spotted fever outbreak among employees of an animal shelter, where the authors, despite the inability to confirm the involved tick species, point out the risk of infection to humans when in close contact with dogs[22].

Another justification is that false positive results would be possible due to other acute infections. Such a situation could explain the titers observed in subjects 1, 2, and 6, who had no epidemiological history. A titer of 1:128 in patient 1 calls attention to the limitation of IFA as an isolated result, without considering the presence or absence of infection risk factors. Moreover, it reinforces the need to consider clinical, laboratorial, and epidemiological evidence in medical care and epidemiological surveillance.

In the patients evaluated, the clinical picture led to suspicion of dengue fever, leptospirosis, and meningococcal disease. This situation has been previously observed in a 2001 report of an investigation carried out in Paulínia, a municipality of the State of São Paulo, where it was observed that these diseases were among the initial diagnostic hypotheses associated with an outbreak of BSF[23]. There is evident difficulty in distinguishing between rickettsial diseases and other pathologies, including dengue fever, especially in endemic periods[24]; leptospirosis; and other febrile diseases, including exanthematic and hemorrhagic fever. Thus, cases of rickettsial diseases may exist, but are not recognized in the municipalities. Indeed, these are considered silent areas for BSF, in which there was intense circulation of dengue virus in 2014.

Moreover, there was not an asymptomatic infection by *R. rickettsii* in Brazil, since *R. rickettsii*is usually associated with severe and highly lethal outcomes; nevertheless, the presence of reactive titers higher than 1:64 may suggest infection. The infection may be due to contact with *Rickettsia* spp. or other Spotted Fever Group rickettsia, including *R. parkeri* infection, as shown in the current study.

The period of increased incidence of transmission by *A. sculptum* is between late April to early May to late September and early October[25]. Confirmed cases were also observed during months in which epidemic periods of dengue fever have been reported[13]. However, in the current study, the IFA-positive samples were collected in different months, mostly in the first semester.

Several study limitations could be highlighted, including serological testing of a single sample, which does not allow for a diagnostic confirmation of BSF according to current criteria. It is noteworthy, however, that just prior to 2004, single samples with IgG titers equal to 1:64 met criteria for laboratory confirmation, which would confirm cases of BSF. Also remarkable is that positive titers of IFA suggested a possible acute infection in the early phase or a previous history of rickettsial diseases in six patients with negative serology for important differential diagnoses. Such information is supported by the fact that they are resident of municipalities where seropositivity in horses for *R. rickettsii* was detected and where the primary host (capybara) and the vector *A. sculptum* were present[12]. In the same study, in municipalities considered endemic to BSF, such as Piracicaba, 54% of investigated horses tested positive. Notably, horses are considered sentinels for the occurrence of human cases, even when tested in a single sample, and they are considered an early indicator of exposure to infected vectors[10]. A study carried out in Pedreira, an endemic municipality in the State of São Paulo, showed 77.3% IFA-positivity in investigated horses[26]. In three regions considered not endemic, there were no horses with reactive IFA sera, further suggesting the role of horses as a sentinel for *Rickettsia* circulation[10]. Nevertheless, some authors suggest that different species of *Rickettsia* can circulate naturally in non-endemic regions without the occurrence of human cases; these would be considered accidental hosts. The method and frequency by which individuals are exposed to transmission foci could justify the absence or low prevalence of the infection in humans[27].

Bragança Paulista, and Atibaia are municipalities that are part of the PCJ river basin. This region presents environmental and ecological characteristics that are propitious for *Rickettsia* circulation and the presence of hosts and vectorsthat favor the occurrence of the disease in humans. Knowing the epidemiological determinants of risk (areas of transmission, type of vegetation, exposure to vectors, and contact with host animals) is essential to increase clinical suspicion and guide surveillance activities. Besides detecting infection in sentinel animals, the evidence of individuals exposed to risk factors with positive serology for *Rickettsia* may ultimately contribute to calling attention to the disease and improving the ability to detect suspected cases.

Expanding the area of serological testing of sentinel animals may increase the awareness and suspicion of BSF in patients suspected of dengue fever, leptospirosis, and meningococcal disease. Suspicion is essential for early, timely, and appropriate laboratory testing (with collection of paired samples) for early diagnostic in endemic and non-endemic areas. Furthermore, other suspected tick-borne diseases should be considered, for which there is no structured surveillance.

In conclusion, based on serological results and reported clinical and epidemiological antecedents, there is evidence of circulation of *Rickettsia* spp. in the studied municipalities, which were not previously classified as being endemic to BSF. Additionally, with consideration of IgM detection among acute febrile patients, the circulation of potentially pathogenic rickettsial species cannot be excluded.

Although a definitive confirmation of human spotted fever cases is not possible with currently established surveillance system criteria, the results presented here may serve as a warning to epidemiological surveillance and health services to improve epidemiological surveillance strategies and increase clinical suspicion for BSF and other rickettsial diseases. In particular, this would ensure the proper case detection and laboratory investigation of patients with possible rickettsial infections that, eventually, may have not been routinely identified.

Acknowledgments

The author acknowledge Valmir Roberto Andrade, *Superintendência de Controle de Endemias- Campinas* and João Fred, *Centro de Vigilância Epidemiológica,* Campinas.

Conflict of interest

The authors declare that there is no conflict of interest.

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