Study of infection by Rickettsiae of the spotted fever group in humans and ticks in an urban park located in the City of Londrina, State of Paraná, Brazil

Estudo da infecção por Rickettsia do grupo da febre maculosa em humanos e carrapatos de um parque urbano na Cidade de Londrina, Estado do Paraná

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ABSTRACT

Introduction: Spotted fevers are emerging zoonoses caused by Rickettsia species in the spotted fever group (SFG). Rickettsia rickettsii is the main etiologic agent of Brazilian spotted fever (BSF) and it is transmitted by Amblyomma spp. ticks. Methods: The study aimed to investigate SFG rickettsiae in the Arthur Thomas Municipal Park in Londrina, PR, by collecting free-living ticks and ticks from capybaras and blood samples from personnel working in these areas. Samples from A. dubitatum and A. cajennense were submitted for PCR in pools to analyze the Rickettsia spp. gltA (citrate synthase gene). Results: All the pools analyzed were negative. Human sera were tested by indirect immunofluorescence assay with R. rickettsii and R. parkeri as antigens. Among the 34 sera analyzed, seven (20.6%) were reactive for R. rickettsii: four of these had endpoint titers equal to 64, 2 titers were 128 and 1 titer was 256. None of the samples were reactive for R. parkeri. An epidemiological questionnaire was applied to the park staff, but no statistically significant associations were identified. Conclusions: The serological studies suggest the presence of Rickettsiae related to SFG that could be infecting the human population studied; however, analysis of the ticks collected was unable to determine which species may be involved in transmission to humans.

Keywords: Amblyomma dubitatum. Amblyomma cajennense. Rickettsia. Epidemiology. PCR.

INTRODUCTION

Spotted fever rickettsioses are caused by bacteria of the genus Rickettsia from the spotted fever group (SFG). R. rickettsii is the etiologic agent of Brazilian spotted fever (BSF) and it is transmitted by Amblyomma spp. tick bites. In Brazil, A. cajennense and A. aureolatum are associated with the transmission of R. rickettsii to humans and animals. In recent decades, BSF has been described in four states of southeastern Brazil, particularly in São Paulo and Minas Gerais. The importance of rickettsiosis is increasing not only due to the identification of new species, but also because its prevalence and distribution are greater than previously suspected. In Brazil, only R. rickettsii has been isolated and characterized in humans and this was only in the State of São Paulo. Other rickettsiae of the SFG have been isolated in ticks, such as R. parkeri and R. bellii, and in fleas, R. felis. Recently, R. parkeri was responsible for causing a mild disease in the United States. In Brazil, no reports of diseases caused by this rickettsia in humans have been recorded; however, it has already been detected in the ticks A. dubitatum and A. triste in the State of São Paulo.

In the State of Paraná, the first case of BSF was notified in the City of São José dos Pinhais, in the southern region of the state, in a man who was infected on a farm. Since 1930, a variety of serological investigative studies on humans, horses and dogs in different regions of the state indicated positive rates of R. rickettsii ranging from 4.7% to 24.1, 5.5% to 55.6% and 1.9% to 22.9%, respectively. There are few confirmed cases of the disease in Paraná, but these data suggest that infection by rickettsiae is occurring in both the human and animal populations.

Despite the lack of any data implicating a specific animal species as a R. rickettsii amplifying host for ticks in Brazil, several studies conducted since the 1930s suggest that capybaras, opossums
and wild rabbits might play this role\textsuperscript{21}. In capybaras (\textit{Hydrochoerus hydrochaeris}), bacteremia is observed for more than 11 days postinoculation\textsuperscript{22} and \textit{A. cajennense} feeding on experimentally infected capybaras later transmit the infection by biting other animals\textsuperscript{23}. Capybaras are hosts to several tick species, including \textit{A. cajennense} and \textit{A. dubitatum}\textsuperscript{22}. Despite the lack of evidence regarding the capacity of \textit{A. dubitatum} to transmit \textit{R. rickettsii}, suspicions exist that it may contribute in the transmission of rickettsiae to humans\textsuperscript{24,25}.

The Arthur Thomas Municipal Park (ATMP) located in the City of Londrina, Paraná, is a landmark for tourism and also has a large number of staff and daily visitors. In a populational dynamics study with free-living ticks performed in this park, a large population of ticks was verified in the vegetation all year round and staff and visitors are constantly exposed to them\textsuperscript{25}. Due to the lack of data concerning the presence of rickettsiae in the parks of Paraná, the objective of this study was to investigate the presence of rickettsiae of the spotted fever group in ticks living in the vegetation and on capybaras in the park and the occurrence of positive serology among staff members.

**METHODS**

**Area studied**

The ATMP is situated in the City of Londrina, State of Paraná, a nonendemic region for BSF. The City of Londrina (23°19'S, 51°10'W) is located in the central-northern region of the State of Paraná, in the southern region of Brazil. It is located at 610m above sea level and has a subtropical climate, with rainfall throughout the year, but with a tendency of concentrating rains during the summer months. The annual average temperature is around 20ºC\textsuperscript{26}.

This park is located within the urban perimeter of Londrina, 6km from the city center and has a total area of 85.47 ha along the middle course of the Cambé River, which forms a dam inside the park. It stands as one of the last areas of Atlantic forest in northwestern Paraná.\textsuperscript{27} The ATMP has diverse fauna, with populations of capuchin monkeys, capybaras, opossums, coatis and agoutis, as well as birds and fishes. The park contains a dam that is surrounded by grass and bushes, where an average of 20 capybaras can be observed for most of the day. In this region, a large number of ticks can be found year round. This place is also commonly visited by the park staff and visitors, which are openly exposed to the attack from these ticks\textsuperscript{25}.

**Ticks from the environment and from capybaras**

In the ATMP, monthly collections of ticks from the environment were conducted over 12 months, from August 2006 to July 2007. Free-living ticks were collected from the park vegetation near areas where capybaras live on the margins of the dam. To collect ticks from the environment, CO\textsubscript{2}-baited traps were used to capture nymphs and adults and drag samplings were used to capture larvae\textsuperscript{28}. Ticks collected from the park were placed in containers with absolute ethanol and transported to the laboratory where they were counted, separated according to developmental stage (larvae, nymph and adult), and where the adults were identified according to the taxonomic key and morphological characteristics\textsuperscript{29,30} and then maintained in absolute ethanol until DNA extraction. Ticks were collected from the capybaras in 2005 by Londrina State University (Universidade Estadual de Londrina, UEL) and Environmental Agency (Secretaria Estadual de Meio Ambiente, SEMA) staff who monitored the capybara population. These ticks were maintained in pure ethanol in the Laboratory of Parasitology and Parasitic Diseases of the UEL. They were identified by the same taxonomic key mentioned above.

In the period of a year, 3,029 adult ticks, 14,186 nymphs and 25,356 larvae were collected from the environment. Regarding adult ticks, a total of 2,526 (81.9\%) were identified as \textit{A. dubitatum} and 503 (18.1\%) as \textit{A. cajennense}\textsuperscript{24} and for ticks collected from capybaras, 40 were identified as \textit{A. dubitatum} and two as \textit{A. cajennense}.

**Blood samples**

In December 2007, blood samples were collected by brachial vein puncture from 34 healthy humans who worked in the park. The samples were stored in sterile tubes, identified and transported to the laboratory, where they were centrifuged (1,500 g for 10min) and serum aliquots were placed into labeled microtubes and stored at -20°C until they were tested by indirect immunofluorescence assay (IFA).

**DNA extraction**

Extraction of DNA and PCR were performed only for adult ticks. For ticks collected from vegetation, a pool of five ticks was defined as a sample unit, resulting in a total of 100 pools of \textit{A. cajennense} and 505 of \textit{A. dubitatum}. The size of the sampling extracted from this population was estimated considering a prevalence of approximately 2\%, which resulted in 78 and 147 pools of \textit{A. cajennense} and \textit{A. dubitatum}, based on statistics and sample design\textsuperscript{31}. All ticks collected from capybaras were submitted for DNA extraction and PCR, consisting of a total of 8 pools of \textit{A. dubitatum} (\textit{n} = 40) and two \textit{A. cajennense} analyzed individually.

Pooled ticks were previously dried at room temperature. With the help of a sterile surgical blade, a longitudinal section was performed in the middle of the tick. Half of the tick was shredded and DNA extraction was performed as previously described\textsuperscript{32} with minor modifications\textsuperscript{33}. The other half of the tick was maintained at -20°C. DNA extraction was performed for each tick and 5µL of each extraction was added to the pool to perform PCR.

**Polymerase chain reaction**

\textit{Initial oligonucleotides (primers)}, \textit{RpCS.877p} (GGGGGCTCTGCTCACGGCGG) and \textit{RpCS.1258n} (ATTGCAAAAAGTACAGTGAAACAA) were used to amplify a 381 base pair region of the \textit{Rickettsia spp. gltA} gene\textsuperscript{34}. For PCR reactions, 2.5µl buffer (10X), 0.2µl deoxynucleoside triphosphates (dNTP 1,25mM), 1.25µl MgCl\textsubscript{2} (50mM), 3µl of each primer (10pmol), 0.3µl Taq DNA polymerase (5,000U/ml), 5µl sample, ultrapure H\textsubscript{2}O (q.s.p. 25µl) were used. Genomic DNA from \textit{A. cajennense} ticks naturally infected with \textit{R. amblyommii} were used as positive controls for PCR reactions and DNA extraction. As a negative control, sterile ultra pure water was used. The stages and conditions of amplification were: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 20sec, annealing at 48°C for 30 sec, elongation at 60°C for 2 min and final elongation at 60°C for 10 min\textsuperscript{35}.

Amplified products were separated in 1.5% TBE (89mM Tris-borate, 2mm EDTA, pH 8) agarose gels using 100bp ladders as size markers (100bp DNA ladder; Life Technologies, Invitrogen, Carlsbad, CA, USA). These bands were compared to a standard 123pb molecular weight marker. Gels were visualized with ethidium bromide under UV illumination and photographed.

**Indirect immunofluorescence assay**

All human serum samples were submitted for IFA in the Department of Preventive Veterinary Medicine and Animal Health.
of University of São Paulo (VPS/USP). Slides were prepared as previously described35 using two Rickettsia species: *R. rickettsii* (strain Tsaiyau32 and *R. parkeri* (strain At24)12. On each slide, a serum previously shown to be nonreactive (negative control) and a known reactive serum (positive control) were tested. Slides were read using an epifluorescent microscope (Olympus, Japan) at 400x magnification and only sera presenting titers against IgG ≥ 64 were considered positive. Reactions were interpreted as previously described35.

**Statistical analysis**

An epidemiological questionnaire was applied to each subject who submitted blood. To evaluate variables, the Chi square or Fisher exact test and odds ratio calculation with 95% confidence intervals of were used. P values < 0.5 were considered significant. Calculations were performed using the Epip program (CDC/Atlanta).

**Ethical considerations**

Collection of human blood was approved by the Research Ethics Committee in Research Involving Human Beings of the UEL (protocol no. 125/07).

## RESULTS

**Tick PCR**

A total of 225 tick pools were analyzed, including 78 (390 ticks) *A. cajennense* and 147 (735 ticks) *A. dubitatum*, collected from the environment. Among the ticks collected from capybaras, 8 *A. dubitatum* pools (40 ticks) were analyzed and 2 *A. cajennense* were analyzed individually. All pools were negative, including those of the individually analyzed ticks. Positive controls produced bands at the expected locations; negative controls did not produce any bands.

**Indirect immunofluorescence assay**

A total of 34 serum samples were collected from humans. All sera were tested by IFA using *R. rickettsii* and *R. parkeri* antigens. Of the 34 total sera analyzed, 7 (20.6%) reacted against *R. rickettsii* antigen at titers ≥ 64. Four of these had endpoint titers equal to 64, 2 titers were 128 and 1 titer was 256. When *R. parkeri* antigen was used, all were negative.

**Epidemiological questionnaire**

Among the 34 subjects who submitted blood samples, 26 (76.5%) affirmed having been bitten by ticks and of these, 9 (34.6%) affirmed that they had acquired tick bites only inside the park, while 12 (46.2%) affirmed having been bitten by ticks inside the park and in other places; 18 (52.9%) lived in a rural area, 21 (61.8%) had worked or were working with animals in a rural area; 15 (44.1%) worked in the park, in jobs involving gardening, security and environmental police, in direct contact with areas infested by ticks.

Among the 7 individuals with reactive serology, 4 (57.1%) affirmed having acquired tick bites in the park and in other areas, two (28.6%) affirmed having acquired tick bites only in the park; 5 (71.4%) lived or worked in rural areas; 3 (42.9%) worked in the park in direct contact with areas infested by ticks and 4 (57.1%) had jobs outside the park or within the administrative block. There were no significant associations between the presence of *R. rickettsii* antibodies and the variables evaluated. None of the individuals investigated reported any clinical manifestations related to the BSF.

## DISCUSSION

Regarding the 2 tick species collected from vegetation, *A. dubitatum* represented 81.9% of adult ticks and was much more prevalent than *A. cajennense*. As capybaras are the primary hosts for *A. dubitatum* and *A. cajennense*, an increased abundance of these species occurs in areas where these animals are established36. Reports of capybaras with positive serology for *R*. *kickettsiae* and reports of these animals with rickettsiaemia have been published. The circulation of *R. rickettsii* in capybaras was observed for more than 11 days, while a separate study showed that it was possible to infect *A. cajennense* by feeding on experimentally infected capybaras and that these infected ticks could transmit the infection to other animals22,23. These data suggest a potential role for capybaras in the BSF cycle and in the cycles of other rickettsiae.

Following analysis of the sample used in this study, the infection rate in the tick population is below 2%. For greater prevalence, at least one pool had to test positive. This infection rate is similar to the rates determined in other works that were performed in nonendemic areas. In the USA, *D. variabilis* ticks from regions where rocky mountain spotted fever (RMSF) has never been reported showed infection rates of 0.7%38. However, the authors also affirmed that the tick infection rate does not vary from that observed in nonendemic areas and regions where cases of human RMSF have occurred39. This emphasizes the importance of serological evaluations in animals and humans to investigate the transmission of *Rickettsia*. Infection by *Rickettsiae* in *A. cajennense* collected from endemic and nonendemic areas in the State of São Paulo showed no positive results by PCR35. However, after the results were analyzed statistically, in one farm, 206 ticks (the smallest sample) were tested and the prevalence of *A. cajennense* infected by *Rickettsia* was estimated at most 1.4% (upper limit of 95% confidence interval). Similarly, in other farm, where 353 ticks were tested (the largest sample), the prevalence was at most 0.8% (upper limit of 95% confidence interval). Thus, the authors concluded that the incidence of rickettsiae-infected ticks was no more than 0.8% to 1.4%.

Other studies report the presence of SFG rickettsiae in *A. dubitatum* in the city of Pedroire, State of São Paulo 11,24 and concluded that this tick could be an important species in the epidemiology of BSF. Some studies have also reported the presence of *R. bellii* in these ticks. Labruna et al11 verified 40% of ticks infected by *R. bellii*, values very similar to those determined by Pacheco38 in the same area. The latter concluded that if some SFG rickettsiae were circulating in the population studied, the ratio of infected ticks would be lower than 0.36% and also that the high proportion of ticks infected by *R. bellii* could inhibit the establishment of other *Rickettsia* species, owing to rickettsial interference that precludes ovarian infection by more than one rickettsia15,40.

In this study, seroprevalence in humans was of 20.6% using *R. rickettsii* antigen. Although no Rickettsiae was identified in the ticks nor were any BSF cases notified in the Londrina region, this rate of seropositivity for people who work in an area at risk of tick bites is considered high. Studies developed in the southeastern region of Brazil verified no reactions or lower seroprevalences (2.8% - 5.3%) using IFA and *R. rickettsii* antigen, for human sera from endemic and nonendemic areas1,5,33,41. In contrast, other serological studies involving humans in 5 different areas of the State of São Paulo, 4 of which are considered endemic and 1 nonendemic15,
verified *R. rickettsii* seroprevalences that varied from 10.1% to 19%. Seroprevalence in the nonendemic area was of 17.8%, similar to the results of the present study. The difference was that in the study conducted in the State of São Paulo, sera also reacted when tested with other antigens, such as *R. parkeri* and *R. felis*. In the present study, no serum reactions occurred with *R. parkeri*. This alone does not indicate that *R. rickettsii* is responsible for the immune response generated in the seropositive subjects, only that it was generated by a SFG rickettsia.

Another serological investigation on humans realized in rural areas of two towns neighboring Londrina revealed antibody rates for *R. rickettsii* similar to those verified in this study: 9.4% in the city of Arapongas and 24.1% in Alvorada do Sul\(^8\). The differential diagnosis for BSF is difficult, when comparing with other diseases commonly identified in the Londrina area, such as leptospirosis and dengue fever. Given this fact, the number of genuine positive cases may be underestimated, since less severe cases may be misdiagnosed or diagnosed inconclusively.

Through the application of the epidemiological questionnaire, it was possible to observe that the majority of humans, regardless of serological results, reported tick bites inside the park and in other areas. Moreover, many of these individuals currently work or have worked with animals in rural areas. Related to this finding and mainly due to the fact that there were no positive ticks identified in the PCR, it is difficult to clearly establish a relation between human infection and the ticks present in the park, though this does not preclude the existence of rickettsia-infected ticks in the area.

Future studies must include other vertebrate hosts that inhabit the park and surveys of a wider diversity of ticks; moreover, serological tests using a broader range of antigens or more specific genetic methods could help to more clearly define the presence or absence of rickettsial activity within the park.

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**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

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