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Rickettsia parkeri: A RICKETTSIAL PATHOGEN TRANSMITTED BY TICKS IN ENDEMIC AREAS FOR SPOTTED FEVER RICKETTSIOSIS IN SOUTHERN URUGUAY

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SUMMARY

At first *Rickettsia conorii* was implicated as the causative agent of spotted fever in Uruguay diagnosed by serological assays. Later *Rickettsia parkeri* was detected in human-biting *Amblyomma triste* ticks using molecular tests. The natural vector of *R. conorii*, *Rhipicephalus sanguineus*, has not been studied for the presence of rickettsial organisms in Uruguay. To address this question, 180 *R. sanguineus* from dogs and 245 *A. triste* from vegetation (flagging) collected in three endemic localities were screened for spotted fever group (SFG) rickettsiosis in southern Uruguay. Tick extracted DNA pools were subjected to PCR using primers which amplify a fragment of the rickettsial *gltA* gene. Positive tick DNA pools with these primers were subjected to a second PCR round with primers targeting a fragment of the *ompA* gene, which is only present in SFG rickettsiae. No rickettsial DNA was detected in *R. sanguineus*. However, DNA pools of *A. triste* were found to be positive for a rickettsial organism in two of the three localities, with prevalences of 11.8% to 37.5% positive pools. DNA sequences generated from these PCR-positive ticks corresponded to *R. parkeri*. These findings, joint with the aggressiveness shown by *A. triste* towards humans, support previous data on the involvement of *A. triste* as vector of human infections caused by *R. parkeri* in Uruguay.

KEYWORDS: *Rhipicephalus sanguineus*; *Amblyomma triste*; *Rickettsia parkeri*; Spotted fever group (SFG); Uruguay.

INTRODUCTION

In the beginning of the 21st century, *Rickettsia rickettsii*, the agent of the Rocky Mountain spotted fever (RMSF) was the only *Rickettsia* known in ticks collected in South America[11]. This organism has been detected in Argentina, Brazil, Canada, Colombia, Costa Rica, the USA, México and Panama, and causes fatal cases in the majority of these countries[11]. Based on development of new molecular biology tools, other rickettsiae have been described in ticks from South America during the last few years[11,26]. The history of spotted fever group (SFG) rickettsiosis in Uruguay is rather complex. The first three autochthonous cases of human rickettsiosis were diagnosed by Indirect Fluorescent Antibody Test (IFAT)[5]. *Rickettsia conorii,* the agent of Mediterranean spotted fever (MSF) was implicated as the causative agent, and the tick *Amblyomma maculatum* was found attached to at least one of the patients[5]. The natural vector and potential reservoir of *R. conorii* in the Old World is the *R. sanguineus* group, implicated in the transmission of other *Rickettsia* species affecting humans[23,34]. In Argentina, *Rickettsia massiliae* was detected in *R. sanguineus* collected in the city of Buenos Aires, and recently a human case of *R. massiliae* infection has been confirmed in the same country[3,9].

In Uruguay, *R. sanguineus* is the second most frequently identified tick involved in bites of humans[31]. However, the infection by *R. conorii* has been neither reported in autochthonous form in the New World nor associated to ticks of the genus *Amblyomma*[19,23]. In Uruguay new human cases have been confirmed suggesting that *Amblyomma triste* tick is the vector (not *Amblyomma maculatum*, a tick species absent in Uruguay) and making the disease considered as "endemic and emergent" in this country[4].

Rickettsia parkeri has been reported as a pathogen of humans in the United States, where it is transmitted by *A. maculatum*[17]. *A. triste* has been found infected with *R. parkeri* in Uruguay[16,32]. In addition to the USA and Uruguay, *R. parkeri* has been also detected in *A. triste* from Brazil, Argentina and in *Amblyomma tigrinum* from Bolivia[14,25,30]. Recently in Argentina, nine cases of SFG rickettsiosis associated to *R. parkeri* infection have been reported. Two of them were confirmed by PCR[21]. In Uruguay, new cases of human rickettsiosis confirmed by serological antibody-absorption tests with purified antigens of *R. parkeri* associated with immunofluorescence assays indicated that the patients were infected by *R. parkeri*[6].

A. triste is the tick species most frequently implicated in human bites in Uruguay and it is particularly aggressive[31].

The main aim of this study was to evaluate the prevalence of *Rickettsia* spp. in ticks collected in a region considered as endemic for human SFG rickettsiosis in Uruguay[4].

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MATERIALS AND METHODS

Studied area and collection of ticks. A total of 245 *A. triste* ticks (155 females and 90 males) were collected from August to October (adult activity season) 2004, by flagging on vegetation in three suburban areas in southern Uruguay, two of them in Canelones county (Toledo Chico, at 34º44´S, 56º06´W, and Escuela Militar de Aeronáutica - EMA, at 34º44´S, 55º58´W), and the other one in Montevideo county (Villa García, at 34º46´S, 56º02´W) (Fig. 1). Details of captures of questing ticks are included in Table 1. Furthermore, 180 *R. sanguineus* ticks (88 females, 64 males, 24 nymphs and four larvae) were collected on dogs in urban and suburban areas from November 2000 to February 2005 in Montevideo and Canelones counties. These samples were obtained after the examination of dogs in rural areas and in private veterinary clinics, as well as from arthropods submitted to the Department of Parasitology, Veterinary Faculty, Montevideo (Uruguay) for diagnosis.

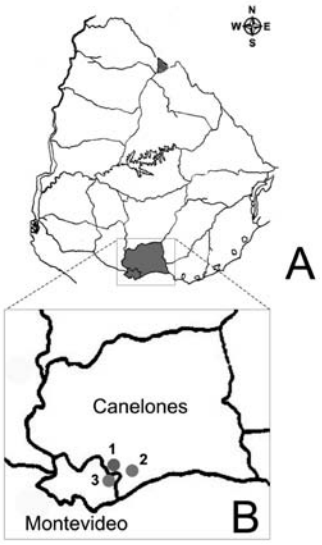

Fig. 1. - A - Map of Uruguay. B - Map of study area magnified, localities of captures: 1. Toledo Chico, Canelones county. 2. Escuela Militar de Aeronáutica, Canelones county. 3. Villa García, Montevideo county.

Ticks were immediately fixed and stored in tubes containing ethanol 70º to preserve DNA, and identified using standard taxonomic keys[1,7].

DNA extraction and polymerase chain reaction (PCR) assays. Ticks were rinsed with distilled water for 10 minutes and dried on sterile filter paper in a laminar-flow hood. Specimens were longitudinally cut, and the DNA of five "half-tick" pools was extracted using the JETQUICK Tissue DNA Spin Kit (GENOMED). The other half of each tick was stored for other studies on pathogen and symbiont.

Specimens were processed by PCR assays using primers CS-78 (forward) (5'-GCAAGTATCGGTGAGGATGTAAT-3') and CS-323 (reverse) (5'-GCTTCCTTAAAATTCAATAAATCAGGAT-3'), which amplify a 401 bp fragment of the citrate synthase gene (*gltA*), previously reported as adequate for detection of *Rickettsia* spp.[12]. PCR cycling conditions were as follows: an initial three min. denaturation cycle at 95 °C followed by 40 cycles of denaturation (95 °C for 15 s), annealing (48 °C for 30 s), and extension (72 °C for 30 s) with a final seven min extension at 72 °C. Distilled water and DNA from *Rickettsia slovaca* (a rickettsial species not present in Uruguay to minimize contamination risks) were used as negative and positive controls, respectively. Ten microliters of the PCR product were separated by electrophoresis in a 1% agarose gel, stained with ethidium bromide, and examined by UV transillumination.

Tick DNA pools found to be positive with primers CS-78 and CS-323 (*gltA*) were subjected to a second PCR round with primers Rr190.70p (forward) (5'-ATGGCGAATATTTCTCCAAAA-3') and Rr190.701n (reverse) (5'-GTTCCGTTAATGGCAGCATCT-3'), which amplify a 631 bp fragment of the *ompA* gene[22]. Conditions for these procedures were an initial three min denaturation cycle at 95 °C, followed by 40 cycles of denaturation (95 °C for 20 s), annealing (46 °C for 30 s), and extension (63 °C for 60 s) with a final seven min extension at 72 °C. Controls were as previously described.

Sequencing and phylogenetic analysis. Nucleotide sequences of PCR products for *gltA* and *ompA* were compared with those available in GenBank using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/blast). Phylogenetic analyses were conducted using *MEGA* version 4.0[29]. A Neighbor-Joining[24] tree was generated using Tajima-Nei method[28] and gaps were excluded in the pairwise comparison. Support for the NJ topology was tested by bootstrapping over 1,000 replications[8].

RESULTS

A total of 49 pools of *A. triste* (31 pools of females and 18 of males) were analyzed. Eight out of 49 pools showed positive PCR results for *gltA* and *ompA* genes. The prevalence of *Rickettsia* in ticks using PCR primers CS-78 / CS-323 and Rr190.70p / Rr190.701n is shown in Table 1, and expressed as percentage and minimum infection rate (MIR) or the minimum percentage of ticks in a pool with detectable *Rickettsia*. This

Table 1
Adults of *Amblyomma triste* ticks collected in southern Uruguay and percentage and minimum infection rate (MIR) of positive *A. triste* pools obtained by PCR using primers for *gltA* and *ompA* gene for *Rickettsia* species

Locality	*Amblyomma triste* ticks				
	N[a]	Female pools	Male pools	Total pools	MIR
EMA	85 (60F, 25M)	0/12 (0.0%)[b]	2/5 (40.0%)	2/17 (11.8%)	2/85 (2.3%)
Toledo Chico	80 (55F, 25M)	3/11 (27.3%)	3/5 (60.0%)	6/16 (37.5%)	6/80 (7.5%)
Villa García	80 (40F, 40M)	0/8 (0.0%)	0/8 (0.0%)	0/16 (0.0%)	0/80 (0.0%)
Total	245 (155F, 90M)	3/31 (9.7%)	5/18 (27.8%)	8/49 (16.3%)	8/145 (5.5%)

[a]N: Number of collected ticks; F: Females, M: Males; [b]Number of tick positive pools / number of tested pools (percentage of positive pools).

calculation is based on the assumption that a PCR-positive pool contains only one positive tick[2,13]. It is interesting to note the high variability in infection rates of questing ticks between localities, with a maximum of 7.5% MIR and 37.5% infected pools in Toledo Chico. The *ompA* nucleotide sequence (462 bp) was deposited in Genbank (accession number JN664898), and it was named *R. parkeri* Canelones. The alignment of our sequence with 19 partial o*mpA* sequences from SFG rickettsiae available in GenBank resulted in a total of 450 sites including gaps. Our sequence grouped with sequences of *R. parkeri* obtained in several countries and *R. parkeri* (strain Cooperi) and *Rickettsia* sp. for *Amblyomma nodosum* in the phylogenetic tree based on these partial *ompA* sequences, but it is separated from other species of *Rickettsia* detected in ticks in South America, as well as *R. conorii* (Fig. 2).

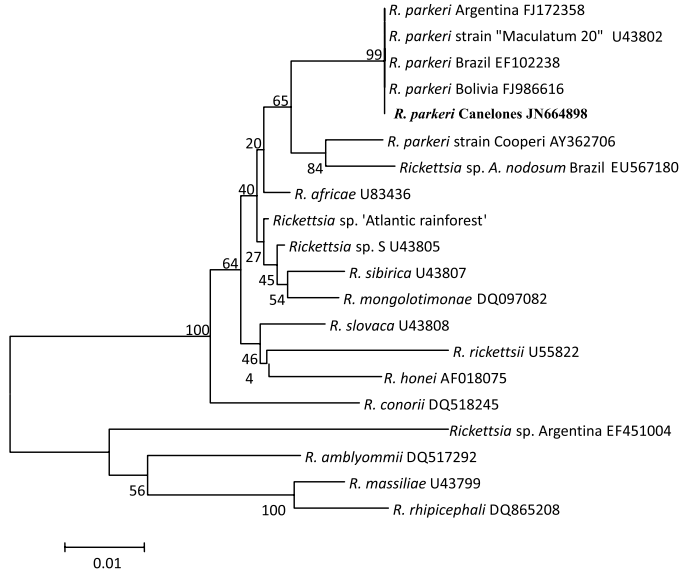

Fig. 2 - Phylogenetic tree based on partial *ompA* gene sequences (450 bp) showing the position of *Rickettsia parkeri* Canelones and selected members of the genus *Rickettsia*. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Tajima-Nei method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. Numbers represent the percentage of 1000 bootstrap replicates supporting each branch. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The GenBank accession number for each sequence is indicated.

Rickettsial DNA was not found in any of the 36 pools prepared with the 180 *R. sanguineus* ticks, using CS-78 and CS-323 primers for *gltA* gene.

DISCUSSION

R. parkeri has been the only pathogen detected in *A. triste* ticks collected in a region considered endemic for human SFG rickettsiosis in Uruguay. In our study, all *R. sanguineus* tested showed negative PCR results, and neither *R. conorii* nor any other *Rickettsia* species was detected. Nevertheless, our data do not rule out that the population of *R. sanguineus* of Uruguay harbors *Rickettsia* spp. These findings further support that *R. parkeri* is the most frequently found SFG rickettsia circulating in this region, *A. triste* being the main vector. It seems that *R. parkeri* has been a neglected pathogen in wide areas of the American continent. It was unequivocally associated to a case of human disease in Virginia (USA) and proved to be transmitted by *A. maculatum*[17]. Since the first association with human disease, more cases have been confirmed in humans in Virginia, Mississippi and probably other states of the USA[18,35]. A recent survey on the range of *R. parkeri* in the USA shows that it is within the known range of the vector *A. maculatum* with prevalence varying according to the different states, averaging 11% of collected ticks in Florida, Georgia, Kentucky, Mississippi and Oklahoma[27]. Additional work carried out in Uruguay, Brazil and Argentina demonstrated variable rates of infection by *R. parkeri* (2.5-9.7%) in *A. triste*, also suggesting that *R. parkeri* may be relatively common in populations of *A. triste*[14,25,32]. By comparison, prevalence of infection of tick vectors with *R. rickettsii* is typically much lower, from 0.05% to 1.3%, as determined by surveys in the USA and Brazil[20,27].

The reasons for differences in tick infection rates among the three studied localities in this work are unknown. All three regions are socially and ecologically similar and are in an endemic area for rickettsiosis.

The first reports implicating *R. conorii* as the etiological agent of human rickettsiosis in the area of study are unsupported by our results. Previous studies on human cases in Uruguay seem to be biased due to crossed reactions using a commercial IFAT kit[5]. To date, autochthonous cases of *R. conorii* infection have not been confirmed in the Americas[19,23].

Previous efforts to detect *Ehrlichia* spp. and *Anaplasma* spp. DNA by PCR in both *R. sanguineus* and *A. triste* in the same area were unsuccessful[33].

Recent research has pointed out the presence of different strains of *R. parkeri* in some domestic and wild animals in Brazil, in areas where *A. triste* is absent[10,15,26]. These studies indicate that other tick species that replace *A. triste* in areas of Brazil (such as *Amblyomma dubitatum*, *A. nodosum* and *A. ovale*) might be involved in the transmission of this pathogen. Of these three ticks species only *A. dubitatum* is recorded for Uruguay. Further assessment is required to understand the role of other tick species in the spread and maintenance of *R. parkeri* in the Neotropics.

RESUMEN

Rickettsia parkeri: patógeno rickettsial transmitido por garrapatas en áreas endémicas de rickettsiosis por fiebre manchada en el sur de Uruguay

Inicialmente, *Rickettsia conorii* fue señalada como el agente causal de la fiebre manchada en Uruguay, diagnosticada mediante pruebas serológicas. Posteriormente, *Rickettsia parkeri* fue detectada mediante técnicas moleculares en garrapatas *Amblyomma triste* colectadas sobre humanos. El vector natural de *R. conorii*, *Rhipicephalus sanguineus*, no ha sido estudiado en cuanto a rickettsias en Uruguay. Para abordar este tema, 180 *R. sanguineus* fueron colectados sobre perros y 245 *A. triste* sobre vegetación en tres localidades consideradas endémicas para fiebres manchadas en el sur de Uruguay. El ADN de las garrapatas fue extraído en pools y sometido a una primera PCR utilizando cebadores que amplifican un fragmento del gen *gltA*, presente en prácticamente todas las especies de *Rickettsia*. Las muestras positivas fueron sometidas a una segunda PCR con cebadores que amplifican un fragmento del gen *ompA,* presente sólo en rickettsias del grupo de las fiebres manchadas (GFM). No se detectó ADN rickettsial en *R. sanguineus*. Sin embargo, muestras de *A. triste* fueron positivas a rickettsiales en dos de las tres localidades estudiadas, con prevalencias de pools positivos del 11.8 y 37.5% respectivamente. La

secuenciación del ADN evidenció la presencia de *R. parkeri*. Basados en estos resultados junto a los anteriores y la agresividad de *A. triste* hacia los humanos, se concluye que esta garrapata es vector de rickettsiosis humana por *R. parkeri* en Uruguay.

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