Ticks and Tick-borne Diseases 5 (2014) 924–927

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

In vitro isolation and infection intensity of *Rickettsia parkeri* in *Amblyomma triste* ticks from the Paraná River Delta region, Argentina

Lucas D. Monje[a,*], Santiago Nava[b], Leandro R. Antoniazzi[a], Valeria C. Colombo[a], Pablo M. Beldomenico[a]

[a] *Laboratorio de Ecología de Enfermedades, Instituto de Ciencias Veterinarias del Litoral, Universidad Nacional del Litoral – Consejo Nacional de Investigaciones Científicas y Técnicas, Esperanza, Argentina*
[b] *Instituto Nacional de Tecnología Agropecuaria, E.E.A. Rafaela, Rafaela, Argentina*

ARTICLE INFO

Article history:
Received 26 February 2014
Received in revised form 11 June 2014
Accepted 13 July 2014
Available online 10 August 2014

Keywords:
Rickettsia parkeri
Amblyomma triste
Isolation
Infection intensity
Argentina

ABSTRACT

In the present study, we report the first in vitro isolation and infection intensity of *Rickettsia parkeri* in *Amblyomma triste* ticks from Argentina. No genetic differences in the molecular targets evaluated were found between *R. parkeri* isolates from Argentina and those *R. parkeri* isolates reported in Uruguay and Brazil, both obtained from *A. triste*. Only a minor difference was observed when compared to *R. parkeri* isolated from *Amblyomma maculatum* from United States. Moreover, the prevalence of infection by *R. parkeri* in ticks collected from the vegetation in the Paraná Delta was high (20.4%). Interestingly, the distribution of *R. parkeri* infection intensity observed in *A. triste* ticks was distinctly bimodal, with approximately 60% of the infected ticks presenting high rickettsial loads (3.8×10^5–4.5×10^7 *ompA* copies/tick) and the remainder with low rickettsial levels (5.6×10^1–6.5×10^3 *ompA* copies/tick). This bimodality in *R. parkeri* infection intensity in ticks could determine differences in the severity of the disease, but also be important for the infection dynamics of this pathogen. Further research exploring the distribution of rickettsial infection levels in ticks, as well as its determinants and implications, is warranted.

Introduction

Amblyomma triste (Acari: Ixodidae) is a tick distributed in the Neotropical and Nearctic regions, but most records are from South America (Guglielmone et al., 2013, 2014). In Argentina, *A. triste* became a tick of public health relevance after the detection of *Rickettsia parkeri* (Nava et al., 2008; Cicuttin and Nava, 2013) and reports of clinical cases of rickettsiosis in humans with a history of tick bites in localities where *A. triste* prevails (Romer et al., 2011). Furthermore, Romer et al. (2011) identified *R. parkeri* DNA in cutaneous lesions in two of the clinical cases documented in the Paraná River Delta region.

Rickettsia parkeri was previously isolated from *A. triste* in Uruguay (Pacheco et al., 2006) and Brazil (Silveira et al., 2007). In Uruguay, human disease allegedly caused by *R. parkeri* has been recurrently reported since 1990 (Conti-Diaz et al., 1990, 2009; Venzal et al., 2004) but it was not until recently that a case of *R. parkeri* infection in a human was confirmed (Portillo et al., 2013). In Brazil, surprisingly, not a single human case of rickettsiosis associated with *A. triste* bite has been reported so far, in spite of the presence of *R. parkeri*-infected *A. triste* ticks (Silveira et al., 2007). However and notwithstanding all the information available about the distribution of this bacterium, there are no previous reports inquiring about the infection levels of *R. parkeri* in naturally infected *A. triste* ticks.

In this report, we describe the isolation and genetic characterization of *R. parkeri* from *A. triste* ticks collected in a region where cases of human *R. parkeri*-rickettsiosis were confirmed. In addition, the intensity of *R. parkeri* infection in naturally infected *A. triste* ticks was estimated.

Materials and methods

Tick collection, identification and processing

Questing adult ticks were collected from the vegetation by drag flagging in fields in the vicinity of Campana city (34°9.5′S, 58°51.8′W), Buenos Aires Province, Argentina. This location corresponds to the Paraná River Delta region, which is the southern

* Corresponding author at: Instituto de Ciencias Veterinarias del Litoral, R.P. Kreder 2805, Esperanza, Santa Fe 3080, Argentina. Tel.: +54 3496 420639x111–353; fax: +54 3496 426304.
E-mail address: lmonje@fcv.unl.edu.ar (L.D. Monje).

http://dx.doi.org/10.1016/j.ttbdis.2014.07.002
1877-959X/

Table 1
Sequences of primers used for amplification of rickettsial genes and tick 16S rRNA.

Gene	Primer	Primer sequence (5′ → 3′)	Fragment size (bp)	Reference
ompA real time	RR190.547	CCTGCCGATAATTATACAGGTTTA	158	Eremeeva et al. (2003)
	RR190.701	GTTCCGTTAATGGCAGCAT		Roux et al. (1996)
gltA	CS78	GCAAGTATCGGTGAGGATGTAAT	350	Labruna et al. (2004)
	CS323	GCTTCCTTAAAATTCAATAAATCAGGAT		Labruna et al. (2004)
gltA	CS239	GCTCTTCTCATCCTATGGCTATTAT	788	Labruna et al. (2004)
	CS1069	CAGGGTCTTCGTGCATTTCTT		Labruna et al. (2004)
ompA	RR190.70	ATGGCGAATATTTCTCCAAAA	592	Regnery et al. (1991)
	RR190.701	GTTCCGTTAATGGCAGCAT		Roux et al. (1996)
ompA nested	RR190.107	GCTTTATTCACCACCTCAAC	435	Kidd et al. (2008)
	RR190.602	AGTGCAGCATTCGCTCCCCCT		Regnery et al. (1991)
ompB	120.M59	CCGCAGGGTTGGTAACTGC	813	Roux and Raoult (2000)
	120.807	CCTTTTAGATTACCGCCTAA		Roux and Raoult (2000)
htrA	17k-5	GCTTTACAAAATTCTAAAAACCATATA	497	Labruna et al. (2004)
	17k-3	TGTCTATCAATTCACAACTTGCC		Labruna et al. (2004)
16S rRNA	T16S5	GACAAGAAGACCCTA	215	de la Fuente et al. (2006)
	T16S3	ATCCAACATCGAGGT		de la Fuente et al. (2006)

extension of the Paranaense Province of the Amazon Phytogeographic Dominion, as defined by Cabrera (1994). Collected ticks were brought alive to the laboratory, where they were identified by using standard taxonomic keys (Estrada-Peña et al., 2005). The tick's surface was disinfected in iodine alcohol (10-min immersion), followed by several washes in sterile water. One half of each tick was processed for DNA extraction using a boiling method (Horta et al., 2010) and total DNA concentration and purity was assessed using the SPECTROstar Nano and the MARS Data Analysis Software (BMG Labtech, Germany). The other half was placed in a sterile vial and immediately frozen at −80 °C.

Real-time PCR and conventional PCR amplification

All tick samples were screened for rickettsial infection through testing them individually by a real-time PCR assay using primers RR190.547 and RR190.701 (Table 1), which were designed to amplify a 158-bp fragment of the outer membrane protein A (*ompA*) of Spotted Fever Group rickettsiae. These primers have shown sensitivity down to five copies of the *omp*A gene of *R. rickettsii* (Eremeeva et al., 2003). Rickettsial DNA was quantified by comparison with 10-fold serial dilutions of known plasmids standards in independent assays. DNA levels were normalized using tick 16S rRNA (de la Fuente et al., 2006). Real-time PCRs were performed in an Applied Biosystems StepOne™ thermocycler with 20 μl per reaction, which contained 4 μl of 5× Phire reaction buffer, 200 μM dNTP, 0.4 pM of each primer, 2 μl of 10 × SYBR Green I (Invitrogen), 150 ng of total DNA and 0.4 μl of Phire Hot Start II DNA polymerase (Thermo Scientific). For *ompA* real-time PCR each run consisted of 3 min at 98 °C for initial denaturation, 50 cycles of 5 s at 98 °C and 10 s at 57 °C followed by extension 20 s at 65 °C and included a positive control (100 ng of DNA of *R. parkeri* strain NOD-infected Vero cells) and a negative control using molecular-grade water. For tick 16S rRNA real-time PCR each run consisted of 3 min at 98 °C for initial denaturation, 40 cycles of 5 s at 98 °C and 10 s at 48 °C followed by extension 20 s at 60 °C and included a negative control using molecular-grade water. Product purity was confirmed by dissociation curves, and random samples were subjected to agarose gel electrophoresis. As was previously described for primers RR190.547 and RR190.701 (Eremeeva et al., 2003), samples presenting threshold cycles (C_t) lower than 37 with melting peaks matching the one observed for the positive control were considered as *Rickettsia*-positive. Randomly selected ticks positive for *ompA* real-time PCR were subjected to further amplification of a larger fragment of *ompA* gene by routine PCR using primers RR190.70 and RR190.701. For those samples that presented low levels of rickettsial DNA, a nested PCR using primers RR190.107 and RR190.602 was attempted with 1 μl of the first PCR as DNA template for the second PCR, using the same cycling conditions. Conventional PCRs were performed in an Ivema T-18 thermocycler (Ivema Desarrollos, Argentina) as described elsewhere (Labruna et al., 2004). PCR products were separated by electrophoresis in a 1.5% agarose gel, stained with GelRed™ (Biotium, USA), and examined by UV transillumination.

Isolation of rickettsiae

Isolation of rickettsiae was attempted on four of the *ompA* real-time PCR positive *A. triste* ticks by the shell vial technique as described previously (Labruna et al., 2004). A rickettsial isolate was considered established in the laboratory after at least 3 passages through 75 cm^2 flasks, each reaching an infected-cell level of >90%. For proper molecular characterization of rickettsiae isolated in cell culture, DNA of infected Vero cells was tested by conventional PCR using all the primers described in Table 1, targeting the following rickettsial genes: *gltA*, *ompA*, *ompB* and *htrA*. These four genes have been characterized at the molecular level in most rickettsial species and are important molecular targets for taxonomy (Labruna et al., 2004; Pacheco et al., 2006; Silveira et al., 2007). PCR products were column purified and sequenced directly in both directions using amplifying primers. Sequencing was conducted under BigDye™ terminator cycling conditions and reacted products were run using an Applied Biosystems 3730xl DNA Analyzer.

Results

A total of 201 adults (74 males, 127 females) of *A. triste* were collected from the vegetation during three consecutive days in December 2011. Forty one ticks (20.4%) were found to contain DNA of the rickettsial gene *ompA* by real-time PCR, 25 of which presented high rickettsial concentration (from 3.8×10^5 to 4.5×10^7 *ompA* copies per tick), while the remaining 16 presented much lower rickettsial levels (from 5.6×10^1 to 6.5×10^3 *ompA* copies per tick) (Fig. 1). A larger fragment of *ompA* was amplified using DNA obtained from ticks with high (four samples) and low (two samples, nested PCR) levels of rickettsial DNA, and the resulting nucleotide sequences were 100% identical to the *ompA* sequence of *R. parkeri* (U43802) in all cases (592/592, nested: 435/435). Of the 41 ticks infected with *R. parkeri*, 19 were male and 22 were female, and of these, 14 males and 11 females presented high loads of *R. parkeri*. All samples were positive for 16S rRNA real-time PCR and no amplification was observed in the negative controls ($C_t > 50$).

Isolation assays by the shell vial technique were attempted from four of the *ompA* real-time PCR positive ticks. *Rickettsiae* were

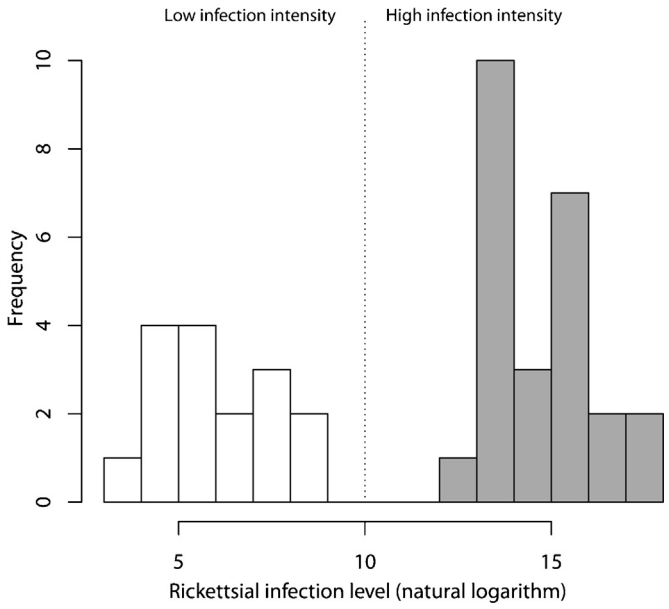

Fig. 1. Distribution of the infection intensity in *Amblyomma triste* ticks positive to *Rickettsia parkeri*.

successfully isolated and established in Vero cell culture from two of the *R. parkeri*-positive ticks (Fig. 2). These isolates were designated At97ARG and At114ARG. DNA extracted from infected cells of the third passage was subjected to PCR targeting the *gltA*, *ompA*, *ompB* and *htrA* genes. PCR products of the expected sizes were obtained for all the genes studied. We sequenced 1106, 592, 817 and 497 nucleotides of the *gltA*, *ompA*, *ompB* and *htrA* genes, respectively. The corresponding gene sequences of the two isolates were 100% identical to each other, and the *gltA*, *ompA*, *ompB* and *htrA* sequences were 100% (1106/1106), 100% (590/590), 100% (817/817) and 99.6% (495/497) similar to the corresponding sequences of *R. parkeri* strain Maculatum from North America (U59732, U43802, AF123717, U17008, respectively), moreover the *htrA* sequence was 100% (497/497) similar to the corresponding sequence of *R. parkeri* isolate At24 from Brazil (EF102237).

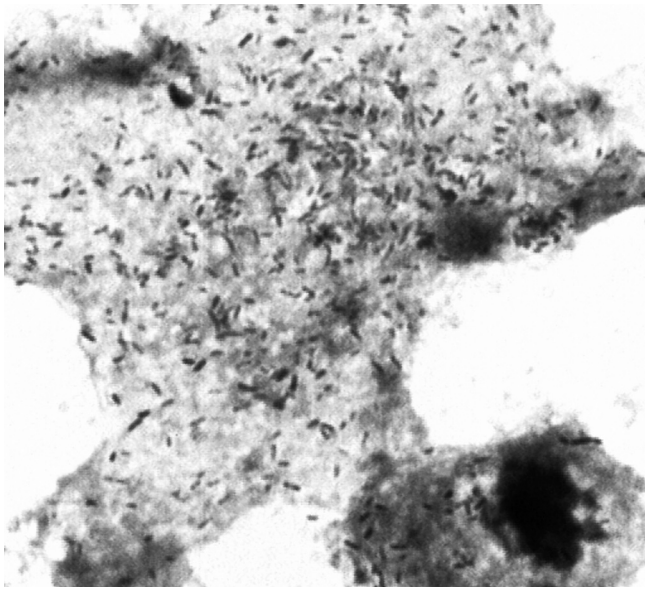

Fig. 2. Vero cells infected by isolate At97ARG rickettsiae as demonstrated by Gimenez staining (Gimenez, 1964). Original magnification 1000×.

Complete identity was also observed with smaller overlapping segments of *gltA* (1093/1093), *ompA* (479/479) and *ompB* (775/775) genes of *R. parkeri* isolate At24 from Brazil (EF102236, EF102238 and EF102239, respectively). Isolation attempts from the other two ticks with positive *ompA* real-time PCR were lost due to bacterial contamination. Partial sequences (*gltA*, *ompA*, *ompB*, *htrA*) from *R. parkeri* isolate At97ARG generated in this study were deposited in GenBank and assigned nucleotide accession numbers KF782319–KF782322, respectively.

Discussion

In the present study, we report the first in vitro isolation of *R. parkeri* from Argentina. *Rickettsia parkeri* isolates At97ARG and At114ARG obtained herein showed no genetic differences with those *R. parkeri* isolates reported in Uruguay (Pacheco et al., 2006) and Brazil (Silveira et al., 2007), both obtained from *A. triste*, and only a slight difference was observed when compared to *R. parkeri* isolated from *Amblyomma maculatum* from the United States. Furthermore, these new isolates were achieved from *A. triste* ticks collected in a region where cases of human *R. parkeri*-rickettsiosis were confirmed (Romer et al., 2011). In Uruguay, human *R. parkeri*-rickettsiosis was only recently confirmed (Portillo et al., 2013). Cases of human rickettsiosis caused by a *Rickettsia* genetically related to *R. parkeri* were recently reported in southern and north-eastern Brazil (Spolidorio et al., 2010; Silva et al., 2011). This *Rickettsia* genotype was designated "Atlantic Rainforest" and was reported infecting *Amblyomma ovale* ticks in Brazil (Szabo et al., 2013). Moreover, further *Rickettsia* genotypes closely related to *R. parkeri* were also described in Brazil, such as *Rickettsia* sp. strain COOPERI infecting *Amblyomma dubitatum* (Labruna et al., 2004) and *R. parkeri* strain NOD infecting *Amblyomma nodosum* ticks (Ogrzewalska et al., 2009), although no cases of human rickettsiosis due to these strains were reported so far. Altogether, these data show that *R. parkeri* isolates obtained from *A. triste* populations throughout South America presented no genetic variation among the gene fragments analyzed so far, and only minor variation was observed when compared to *R. parkeri* isolates obtained from *A. maculatum* from North America. Interestingly, *A. triste* and *A. maculatum* present a very close phylogenetic relationship (Estrada-Peña et al., 2005), but a more distant relationship with those *Amblyomma* species that were reported infected with other *Rickettsia* species genetically related to *R. parkeri*, namely *A. ovale*, *A. dubitatum* and *A. nodosum* (Marrelli et al., 2007; Nava et al., 2009).

Previously, Nava et al. (2008) reported *R. parkeri* infecting 8.4% of the *A. triste* ticks collected from the vegetation in the Paraná River Delta. In the present study, a higher *R. parkeri* infection rate was found in questing ticks (20.4%) in the same location. The differences observed in infection rates could be in part due to inter-annual variation or to the high sensitivity reported for the real-time PCR assay used in our study (Eremeeva et al., 2003), compared with the PCR approach used by Nava et al. (2008). However, we cannot rule out the possibility that infection incidence of *R. parkeri* is indeed increasing, posing a risk for public health. In this respect, further research should be aimed at elucidating whether climatic conditions, soil management, cattle presence or host abundance is affecting *R. parkeri* infection rate of *A. triste* ticks.

Interestingly, the distribution of *R. parkeri* infection intensity observed in *A. triste* ticks was distinctly bimodal, with approximately 60% of the infected ticks presenting high rickettsial loads (3.8×10^5–4.5×10^7 *ompA* copies/tick) and the remainder with low rickettsial levels (5.6×10^1–6.5×10^3 *ompA* copies/tick) (Fig. 1). Although very pertinent for our understanding of the eco-epidemiology of rickettsial pathogens, the distribution of the levels of infection in the ticks has not been previously assessed. The

bimodal distribution found herein could have important epidemiological implications, as the infection intensity in the tick must be directly related with the exposure dose after a tick bite. Elucidating the determinants of this distinct distribution is also crucial to better understand the ecology of this disease (e.g. circumstances that cause a predominance of high infection intensities in ticks would consequently increase the risk of infection in humans). In addition, this bimodality in *R. parkeri* infection intensity in ticks could determine differences in the severity of the disease, and also result in different implications. For example, a previous study conducted with *Leishmania major* (Kimblin et al., 2008) reported that high-dose inoculums of this parasite produced severe pathologies with stern and lasting lesions in the inoculation site that eventually healed, meanwhile low-dose infections produced slight pathologies but with a high persistence of parasites in the inoculation site, establishing these low-dose infected hosts as long-term reservoirs of infection back to the vector. However, whether *R. parkeri* uses a similar mechanism of transmission or not, requires further research. Moreover, uptake of infection by co-feeding ticks at the inoculation site has been previously documented (Zemtsova et al., 2010; Matsumoto et al., 2005).

Recently, *R. parkeri* preservation was demonstrated by transstadial maintenance and trans-ovarial transmission in *A. triste* ticks; however, notable deleterious effects were caused by *R. parkeri* on engorged nymphs (Nieri-Bastos et al., 2013). These data suggests that the tick vector, instead of a vertebrate host, could be acting as a reservoir; however, it cannot be disregarded that the deleterious effect of *R. parkeri* on *A. triste* engorged nymphs would fade out infected ticks from the tick population in a long-term scenario. In this situation, a differential transmission mechanism of *R. parkeri* in *A. triste* ticks infected with low levels of the pathogenic agent could add to the long-term maintenance of this bacterium in nature. Further research exploring the distribution of rickettsial infection levels in ticks, as well as its determinants and implications, is warranted.

Finally, the isolates of *R. parkeri* obtained herein will also provide us with a valuable tool to develop diagnostic tests which will allow to assess the serological status of livestock and wildlife populations and to confirm human cases suspicious of rickettsiosis in our region.

Acknowledgments

The authors would like to thank Dr. Charles Figueroa for plasmid construction, Dr. María Sol Renna and Dr. Marcelo Labruna for cell culture assistance and Cristian Ortiz for field work assistance. This study was supported by ANPCyT (PICT-2008-0090), UNL (CAI+D 501-201101-00498-LI) and International Cooperation Project MINCyT-CAPES (BR/11/08).

References

Cabrera, A.L., 1994. Enciclopedia Argentina de Agricultura y Jardinería. Fascículo 1. Regiones Fitogeográficas argentinas. ACME, Buenos Aires.

Cicuttin, G., Nava, S., 2013. Molecular identification of *Rickettsia parkeri* infecting *Amblyomma triste* ticks in an area of Argentina where cases of rickettsiosis were diagnosed. Mem. Inst. Oswaldo Cruz 108, 123–125.

Conti-Diaz, I.A., Moraes-Filho, J., Pacheco, R.C., Labruna, M.B., 2009. Serological evidence of *Rickettsia parkeri* as the etiological agent of rickettsiosis in Uruguay. Rev. Inst. Med. Trop. Sao Paulo 51, 337–339.

Conti-Diaz, I.A., Rubio, I., Somma Moreira, R.E., Perez, B.G., 1990. Rickettsiosis cutáneo ganglionar por *Rickettsia conorii* en el Uruguay. Rev. Inst. Med. Trop. Sao Paulo 32, 313–318.

de la Fuente, J., Almazan, C., Naranjo, V., Blouin, E.F., Kocan, K.M., 2006. Synergistic effect of silencing the expression of tick protective antigens 4D8 and Rs86 in *Rhipicephalus sanguineus* by RNA interference. Parasitol. Res. 99, 108–113.

Eremeeva, M.E., Dasch, G.A., Silverman, D.J., 2003. Evaluation of a PCR assay for quantitation of *Rickettsia rickettsii* and closely related spotted fever group rickettsiae. J. Clin. Microbiol. 41, 5466–5472.

Estrada-Peña, A., Venzal, J.M., Mangold, A.J., Cafrune, M.M., Guglielmone, A.A., 2005. The *Amblyomma maculatum* Koch, 1844 (Acari: Ixodidae: Amblyomminae) tick group: diagnostic characters, description of the larva of *A. parvitarsum* Neumann, 1901, 16S rDNA sequences, distribution and hosts. Syst. Parasitol. 60, 99–112.

Gimenez, D.F., 1964. Staining rickettsiae in yolk-sac cultures. Stain. Technol. 39, 35–140.

Guglielmone, A.A., Nava, S., Mastropaolo, M., Mangold, A.J., 2013. Distribution and genetic variation of *Amblyomma triste* (Acari: Ixodidae) in Argentina. Ticks. Tick. Borne. Dis. 4, 386–390.

Guglielmone, A.A., Robbins, R.G., Apanaskevich, D.A., Petney, T.N., Estrada-Peña, A., Horak, I., 2014. The Hard Ticks of the World. Springer, Dordrecht.

Horta, M.C., Sabatini, G.S., Moraes-Filho, J., Ogrzewalska, M., Canal, R.B., Pacheco, R.C., Martins, T.F., Matushima, E.R., Labruna, M.B., 2010. Experimental infection of the opossum *Didelphis aurita* by *Rickettsia felis*, *Rickettsia bellii*, and *Rickettsia parkeri* and evaluation of the transmission of the infection to ticks *Amblyomma cajennense* and *Amblyomma dubitatum*. Vector Borne Zoonotic Dis. 10, 959–967.

Kidd, L., Maggi, R., Diniz, P.P., Hegarty, B., Tucker, M., Breitschwerdt, E., 2008. Evaluation of conventional and real-time PCR assays for detection and differentiation of spotted fever group *Rickettsia* in dog blood. Vet. Microbiol. 129, 294–303.

Kimblin, N., Peters, N., Debrabant, A., Secundino, N., Egen, J., Lawyer, P., Fay, M.P., Kamhawi, S., Sacks, D., 2008. Quantification of the infectious dose of *Leishmania major* transmitted to the skin by single sand flies. Proc. Natl. Acad. Sci. U.S.A. 105, 10125–10130.

Labruna, M.B., Whitworth, T., Horta, M.C., Bouyer, D.H., McBride, J.W., Pinter, A., Popov, V., Gennari, S.M., Walker, D.H., 2004. Rickettsia species infecting *Amblyomma cooperi* ticks from an area in the state of Sao Paulo, Brazil, where Brazilian spotted fever is endemic. J. Clin. Microbiol. 42, 90–98.

Marrelli, M.T., Souza, L.F., Marques, R.C., Labruna, M.B., Matioli, S.R., Tonon, A.P., Ribolla, P.E., Marinotti, O., Schumaker, T.T., 2007. Taxonomic and phylogenetic relationships between neotropical species of ticks from genus *Amblyomma* (Acari: Ixodidae) inferred from second internal transcribed spacer sequences of rDNA. J. Med. Entomol. 44, 222–228.

Matsumoto, K., Ogawa, M., Brouqui, P., Raoult, D., Parola, P., 2005. Transmission of *Rickettsia massiliae* in the tick, *Rhipicephalus turanicus*. Med. Vet. Entomol. 19, 263–270.

Nava, S., Elshenawy, Y., Eremeeva, M.E., Sumner, J.W., Mastropaolo, M., Paddock, C.D., 2008. *Rickettsia parkeri* in Argentina. Emerg. Infect. Dis. 14, 1894–1897.

Nava, S., Mangold, A.J., Mastropaolo, M., Venzal, J.M., Oscherov, E.B., Guglielmone, A.A., 2009. *Amblyomma boeroi* n.sp. (Acari: Ixodidae), a parasite of the Chacoan peccary *Catagonus wagneri* (Rusconi) (Artiodactyla: Tayassuidae) in Argentina. Syst. Parasitol. 73, 161–174.

Nieri-Bastos, F.A., Szabo, M.P., Pacheco, R.C., Soares, J.F., Soares, H.S., Moraes-Filho, J., Dias, R.A., Labruna, M.B., 2013. Comparative evaluation of infected and noninfected Amblyomma triste ticks with Rickettsia parkeri, the agent of an emerging rickettsiosis in the New World. Biomed. Res. Int. 2013, 402737.

Ogrzewalska, M., Pacheco, R.C., Uezu, A., Richtzenhain, L.J., Ferreira, F., Labruna, M.B., 2009. Rickettsial infection in *Amblyomma nodosum* ticks (Acari: Ixodidae) from Brazil. Ann. Trop. Med. Parasitol. 103, 413–425.

Pacheco, R.C., Venzal, J.M., Richtzenhain, L.J., Labruna, M.B., 2006. *Rickettsia parkeri* in Uruguay. Emerg. Infect. Dis. 12, 1804–1805.

Portillo, A., Garcia-Garcia, C., Sanz, M.M., Santibanez, S., Venzal, J.M., Oteo, J.A., 2013. A Confirmed Case of *Rickettsia parkeri* Infection in a Traveler from Uruguay. Am. J. Trop. Med. Hyg. 89, 1203–1205.

Regnery, R.L., Spruill, C.L., Plikaytis, B.D., 1991. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J. Bacteriol. 173, 1576–1589.

Romer, Y., Seijo, A.C., Crudo, F., Nicholson, W.L., Varela-Stokes, A., Lash, R.R., Paddock, C.D., 2011. *Rickettsia parkeri* Rickettsiosis, Argentina. Emerg. Infect. Dis. 17, 1169–1173.

Roux, V., Fournier, P.E., Raoult, D., 1996. Differentiation of spotted fever group rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protein rOmpA. J. Clin. Microbiol. 34, 2058–2065.

Roux, V., Raoult, D., 2000. Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer-membrane protein rOmpB (ompB). Int. J. Syst. Evol. Microbiol. 50 (Pt 4), 1449–1455.

Silva, N., Eremeeva, M.E., Rozental, T., Ribeiro, G.S., Paddock, C.D., Ramos, E.A., Favacho, A.R., Reis, M.G., Dasch, G.A., de Lemos, E.R., Ko, A.I., 2011. Eschar-associated spotted fever rickettsiosis, Bahia, Brazil. Emerg. Infect. Dis. 17, 275–278.

Silveira, I., Pacheco, R.C., Szabo, M.P., Ramos, H.G., Labruna, M.B., 2007. *Rickettsia parkeri* in Brazil. Emerg. Infect. Dis. 13, 1111–1113.

Spolidorio, M.G., Labruna, M.B., Mantovani, E., Brandao, P.E., Richtzenhain, L.J., Yoshinari, N.H., 2010. Novel spotted fever group rickettsiosis, Brazil. Emerg. Infect. Dis. 16, 521–523.

Szabo, M.P., Nieri-Bastos, F.A., Spolidorio, M.G., Martins, T.F., Barbieri, A.M., Labruna, M.B., 2013. In vitro isolation from *Amblyomma ovale* (Acari: Ixodidae) and ecological aspects of the Atlantic rainforest *Rickettsia*, the causative agent of a novel spotted fever rickettsiosis in Brazil. Parasitology 140, 719–728.

Venzal, J.M., Portillo, A., Estrada-Peña, A., Castro, O., Cabrera, P.A., Oteo, J.A., 2004. *Rickettsia parkeri* in *Amblyomma triste* from Uruguay. Emerg. Inf. Dis. 10, 1493–1495.

Zemtsova, G., Killmaster, L.F., Mumcuoglu, K.Y., Levin, M.L., 2010. Co-feeding as a route for transmission of *Rickettsia conorii israelensis* between *Rhipicephalus sanguineus* ticks. Exp. Appl. Acarol. 52, 383–392.