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Seroepidemiological survey of *Rickettsia* spp. in dogs from the endemic area of *Rickettsia parkeri* rickettsiosis in Uruguay

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ABSTRACT

Rickettsia parkeri rickettsiosis is a vector-borne zoonosis that occurs in some countries of the American continent. Following the first description and determination of the pathogenicity to humans in 2004 in USA, this bacterium has been reported in several South American countries. Human cases have been diagnosed in both Uruguay and Argentina in the past years. This study consisted in a serosurvey of 1000 domestic dogs living in the endemic area of rickettsiosis in Uruguay, where *Amblyomma triste* is the tick vector. Sera were analyzed by Indirect Immunofluorescence Assay (IFA), against antigens of three different rickettsial species: *R. rhipicephali*, *R. felis* and *R. parkeri*. It was determined that 20.3% of the dogs had antibodies that reacted to at least one of the three species tested, taking as cut off ≥64 titers. Furthermore, 140 of the seropositive dogs (14%) had a titer at least 4 times higher to *R. parkeri* than those of any of the other species, thus, it was considered that the immune response was stimulated by that species in particular. This is the first serological survey in primary hosts for adults of *A. triste* in Uruguay, and therefore the first prevalence values are reported. Adult *A. triste* ticks collected from the environment as well as from dogs were analyzed by PCR in order to confirm the current circulation of the agent in the area. In this matter, two out of 28 ticks from dogs, and 3 out of 53 ticks from the environment were positive, and the corresponding sequence analysis revealed 100% similarity with *R. parkeri* strain maculatum.

1. Introduction

Rickettsia parkeri is a bacterium belonging to the order Rickettsiales, family Rickettsiaceae. It is included in the spotted fever group (SFG), which comprises all pathogenic tick-borne *Rickettsia* species (Parola et al., 2013). While *R. parkeri* was firstly reported infecting *Amblyomma maculatum* ticks in the United States in 1939 (Parker et al., 1939; Lackman et al., 1949), its pathogenicity to humans was determined only in 2004, in that same country (Paddock et al., 2004). Also in 2004, the presence of *R. parkeri* infecting *Amblyomma triste* ticks was reported in Uruguay (Venzal et al., 2004), and few years later in Brazil (Silveira et al., 2007) and Argentina (Nava et al., 2008). *R. parkeri* has also been found infecting the ticks *Amblyomma tigrinum* from Bolivia, Uruguay, and Argentina (Tomassone et al., 2010; Lado et al., 2014; Romer et al., 2014), and *A. maculatum* from Peru (Flores-Mendoza et al., 2013). Human clinical cases of *R. parkeri*-caused rickettsiosis have been confirmed in several states of southern United States, Uruguay and Argentina (Paddock, 2005; Whitman et al., 2007; Paddock et al., 2008; Conti-Díaz et al., 2009; Cragun et al., 2010; Romer et al., 2011; Portillo et al., 2013; Romer et al., 2014).

The coastal counties of the Rio de la Plata and the Atlantic Ocean are considered the main spotted-fever-endemic area of southern Uruguay, with several laboratory-confirmed clinical

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cases (Conti-Díaz, 2001; Venzal et al., 2012; Portillo et al., 2013). Although information concerning *A. triste* seasonality, distribution, hosts, and infection by *R. parkeri* have been reported from this area (Venzal et al., 2004; 2008, 2012; Pacheco et al., 2006), epidemiological data are still very scarce. Therefore, this study aimed to perform a serological analysis of domestic dogs in southern Uruguay. Because domestic dogs are main hosts for the adult state of *A. triste* in Uruguay, and are reared in close contact with humans, dogs are indeed the most efficient sentinel for Uruguayan spotted fever. Along with the serological study, ticks were collected from dogs and from the environment in order to determine by molecular methods if *R. parkeri* is still circulating in the area.

2. Materials and methods

The current study comprised 4 counties of southern Uruguay: Canelones, Maldonado, Montevideo, and Rocha. In both Canelones and Maldonado, cases of human rickettsiosis have been reported (Conti-Díaz et al., 2009). *R. parkeri*-infected *A. triste* ticks have been reported from Canelones, Maldonado, and Montevideo (Venzal et al., 2004, 2012; Pacheco et al., 2006).

A total of 1000 owned dogs were sampled (blood and tick collection) in sub-urban and urban areas between March 2012 and June 2013, encompassing the four year seasons. Blood samples were transported to the laboratory at room temperature and subsequently centrifuged for 10 min at 1500 × *g*. Then, the serum was stored in freezer at −20 °C until analysis. Sera were tested by an indirect immunofluorescence assay (IFA) using crude antigens derived from *three Rickettsia* species (*R. parkeri* strain At24, *R. felis* strain Pedreira, and *R. rhipicephali* strain HJ#5), as previously described (Horta et al., 2004; Labruna et al., 2007; Saito et al., 2008). *R. parkeri* and *R. felis* are the only two *Rickettsia* species that have been reported in Uruguay (Venzal et al., 2006, 2012). In addition, we used *R. rhipicephali* because it is genetically (and antigenically) close-related to *R. massiliae*, which has been reported infecting *Rhipicephalus sanguineus* ticks collected from dogs in Buenos Aires, Argentina (Cicuttin et al., 2004, 2014), very close to Uruguay.

Canine sera were diluted in twofold increments with PBS starting from a 1:64 dilution. Serum was considered to contain antibodies against rickettsiae if it displayed a reaction at 1:64. Endpoint titers against each *Rickettsia* species were determined by testing serial twofold serum dilutions. On each slide, a serum previously shown to be non-reactive (negative control) and a known reactive serum (positive control) were tested. Fluorescein isothiocyanate anti-dog immunoglobulin G (Sigma) was applied for fixed antigen reaction and visualized by fluorescence microscope. A serum showing a titer for a *Rickettsia* species at least fourfold that observed for any other *Rickettsia* species was considered homologous for the first *Rickettsia* species or a very closely related genotype (Horta et al., 2004; Pinter et al., 2008; Piranda et al., 2008; Saito et al., 2008). Canine seropositivity was compared according the four year seasons by the Chi-Square test. The present study was previously approved by the Research Ethics Committee of the Universidad de la República, Montevideo, Uruguay.

Questing adult ticks were collected from vegetation using cloth flags, in the same localities where dogs were blood sampled. Ticks from the sampled dogs were also collected. All ticks were preserved in 95% ethanol, and taken to the laboratory where they were identified by using standard taxonomic keys (Martins et al., 2014). For molecular analysis to determine rickettsial detection, ticks were processed individually by DNA extraction using a commercial kit (Wizard® Genomic DNA Purification Kit, Promega) following the manufacturer's instructions. DNA extracts were evaluated by using conventional PCR targeting a fragment of the rickettsial citrate synthase gene (*gltA*), as previously described (Labruna et al., 2004). Samples positive in this first PCR were tested in a second PCR targeting a fragment of the rickettsial 190-kDa outer membrane protein gene (*ompA*), which is present only in SFG *Rickettsia* species (Regnery et al., 1991). All *ompA* amplicons were sequenced and compared to corresponding sequences in GenBank by using the BLAST 2.0 program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3. Results and discussion

Among the 1000 dogs, 289 were sampled during summer, 222 during autumn, 214 during winter, and 275 during spring. Overall, 20.3% (203/1000) of the serum samples reacted to at least one of the three *Rickettsia* species tested by IFA. Among the 203 seropositive samples, 92 reacted against only *R. parkeri*, 78 against *R. parkeri* and *R. rhipicephali*, 3 against *R. parkeri* and *R. felis*, and 30 reacted simultaneously to all three *Rickettsia* species. No serum sample reacted to *R. felis* or *R. rhipicephali* without reacting to *R. parkeri*. Serum endpoint titers ranged from 64 to 32,768 for *R. parkeri*, 64 to 2048 for *R. rhipicephali*, and 64 to 512 for *R. felis*. A total of 140 (14.0%) canine sera showed titers to *R. parkeri* at least 4-fold higher than those to any of the other two antigens. The antibody titers in these 140 dogs were considered to have been stimulated by *R. parkeri* or a very closely related species. Canine seropositivity was statistically similar among the four sampled counties (Table 1). Regarding the year seasons, there was a significant accumulation of seropositive dogs in spring (32.0%), over the other seasons (14.9–16.6%) ($P < 0.001$).

A total of 81 *A. triste* adult ticks, 53 from vegetation and 28 from dogs, were collected. Molecular analysis revealed the presence of SFG *Rickettsia* in 6.2% (5/81) ticks through *gltA* and *ompA* PCR amplification. In all 5 cases the *ompA* partial sequences showed 100% similarity to *R. parkeri* strain At24 (GenBank accession number: EF102238), and 100% to *R. parkeri* strain "Maculatum 20" (GenBank accession number: U43802). The 5 *R. parkeri*-infected ticks corresponded to 3 specimens collected from the environment, and 2 specimens collected from two different dogs. Serological analysis of these two dogs showed evidence of antibodies response toward *R. parkeri*, with endpoint titers of 1024 and 4096.

This study reports, for the first time, serologic evidence of rickettsial infection on dogs in Uruguay. IFA is currently the reference method for serological diagnosis of rickettsial infection in humans and animals (Parola et al., 2013). However, cross-reactive antibodies between SFG *Rickettsia* species are often observed but, in some cases, the differences in titers may be great enough to differentiate the rickettsial species potentially stimulating the immune response (Raoult and Roux, 1997; Parola et al., 2005). Overall, 20.3% of the dogs of the present study were seroreactive to *Rickettsia* spp., with at least 140 dogs showing serologic evidence (possibly homologous reaction) of infection caused by *R. parkeri* or a very closely related species. These dogs were from areas where *R. parkeri*-caused human clinical cases and *R. parkeri*-infected ticks were reported (Venzal et al., 2004, 2012; Pacheco et al., 2006; Conti-Díaz et al., 2009). In addition, we confirm that the *A. triste* population of

Table 1
Distribution of seropositive dogs (IgG titers ≥64 by indirect immunofluorescence assay) according to the county where the dogs were sampled in southern Uruguay from March 2012 to June 2013.

Counties	No. seropositive dogs/No. tested*	Seropositivity (%)
Canelones	123/587 a	21.0
Maldonado	40/196 a	20.4
Montevideo	9/76 a	11.9
Rocha	31/141 a	22.0
Total	203/1000	20.3

* Values followed by the same letter in the same column means that the proportion of seropositive dogs was not statistically different between counties ($P > 0.05$).

this area remains infected by *R. parkeri* because we found that 6.2% of the ticks collected from dogs and the environment were infected by this rickettsial agent. Therefore, we can assume that at least part of the seropositive dogs of the present study was exposed to *R. parkeri* infection, most probably, via *A. triste* ticks. This assumption is corroborated by a recent study that demonstrated under laboratory conditions that *A. triste* is a competent vector of *R. parkeri* (Nieri-Bastos et al., 2013).

The present study reports a slightly higher *R. parkeri*-infection rate for ticks collected from dogs (7.1%) than from the environment (5.7%); however, these rates are not significantly different (data not shown). While it is not known if dogs could act as an infection source of *R. parkeri* to *A. triste* ticks, recent studies with a *R. parkeri*-like agent (strain Atlantic rainforest) in Brazil demonstrated that *Amblyomma ovale* ticks collected from dogs had higher infection rates than the same tick species collected from the dog environment, suggesting some kind of rickettsial horizontal transmission from dogs to ticks (Sabatini et al., 2010; Szabó et al., 2013).

The present study found a 20.3% overall prevalence of seropositive dogs. To our knowledge, there has been no serosurveys for rickettsial infection in dogs from areas where *R. parkeri* is known to occur, either in South or North America. However, one recent study in the state of Rio Grande do Sul (southern Brazil), in an area 400–450 km far from the area of the present study, reported a 42.4% overall seroprevalence for SFG *Rickettsia* in dogs, with most of the seropostive dogs displaying higher endpoint titers to *R. parkeri* than to other *Rickettsia* species (Saito et al., 2008). In that Brazilian study, canine seropositivity was statistically associated with dogs living in rural areas and with dogs used for herding or hunting (Saito et al., 2008). Therefore, probably, the lower seroprevalence values of the present study are due to sampling urban and suburban dogs, which are raised in areas where *A. triste* is less prevalent, when compared to rural areas (Venzal et al., 2008). On the other hand, the relevance of testing urban and suburban dogs is because they are exposed to ticks on the environment and lives in close contact to humans.

Under natural conditions in Uruguay, only the adult stage of *A. triste* is known to feed on dogs; larvae and nymphs feed chiefly on small rodents (Venzal et al., 2003, 2008). A seasonal dynamics study of *A. triste* in Uruguay reported that highest activity period of adult ticks occurs during spring (Venzal et al., 2008). In the present study, canine seropositivity was significantly higher in spring than the other seasons of the year, indicating that spring is the season with highest exposure of dogs to *R. parkeri* infection. Because the adult stage of *A. triste* is the one implicated to transmit *R. parkeri* to humans (Conti-Díaz et al., 2009; Venzal et al., 2004), spring is indeed the period of the year where humans are at higher risks of acquiring SFG rickettsiosis in Uruguay. Therefore, health authorities in Uruguay should concentrate efforts to prevent SFG rickettsiosis during this period of the year, in areas where *R. parkeri* is known to occur. Additionally, our study indicates that canine serosurvey should be employed in other to identify and to confirm SFG-endemic areas, since dogs (primary hosts for *A. triste* adult ticks) would act as sentinels for *R. parkeri* infection.

3.1. Conclusions

This study reports, for the first time, serologic evidence of rickettsial infection on dogs in Uruguay, and the first prevalence values are presented. Overall, 20.3% of the dogs of the present study were seroreactive to *Rickettsia* spp., with at least 140 dogs showing serologic evidence (possibly homologous reaction) of infection caused by *R. parkeri* or a very closely related species. Regarding to the seasonality, canine seropositivity was significantly higher in spring than the other seasons of the year, indicating that spring is the season with highest exposure of dogs to *R. parkeri* infection.

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