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Molecular detection of *Rickettsia bellii* and *Rickettsia* sp. strain Colombianensi in ticks from Cordoba, Colombia

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ABSTRACT

The purpose of this study was to provide molecular evidence of *Rickettsia* spp. in ticks collected from 2 sites of Cordoba. From May to June 2009, 1069 *Amblyomma cajennense* ticks were removed from 40 capybaras (*Hydrochoerus hydrochaeris*) in a rural locality of Monteria. Furthermore, 458 *Amblyomma* sp. larvae and 20 *Amblyomma* sp. nymphs were collected in a rural locality of Los Cordobas (Cordoba) by drag sampling on vegetation ($n = 1547$). Ticks were grouped into pools and tested for rickettsial infection by real-time PCR targeting the rickettsial gene *gltA*. Subsequently, PCR targeting for *gltA*, *ompA*, *ompB*, and 16S rRNA, sequencing, and phylogenetic analyses were undertaken. Rickettsial DNA was detected in 10 (4.6%) out of 214 pools of ticks by RT-PCR. Five (33%) of free-living *Amblyomma* sp. larval pools were positive, as well as 5 (2.6%) pools from *A. cajennense*. Only the *gltA* gene was amplified from 5 pools of free-living larvae. The nucleotide sequences were 100% identical to *R. bellii* by BLAST. Only one pool from *A. cajennense* was positive for *gltA*, *ompA*, *ompB*, and 16S rRNA. The partial nucleotide sequences of these genes were 100% identical to nucleotide sequences of the same genes of a new proposed species *Candidatus* Rickettsia sp. strain Colombianensi. This is the first report of *R. bellii* in ticks in Colombia and the second report of detection of *Candidatus* Rickettsia sp. strain Colombianensi. These *Rickettsia* species are still considered of unknown pathogenicity. Further studies are needed to characterize the ecological and potential pathogenic role of these 2 *Rickettsia* species found in Cordoba.

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Introduction

The *Rickettsia* genus includes bacteria of the order Rickettsiales in the α-subdivision of the Proteobacteria class. They are Gram-negative coccobacilli in obligate association with eukaryote cells. A number of species have been identified in various terrestrial arthropods and recently in leeches and amebae (Perlman et al., 2006). Deep phylogenomic analyses illustrate the wide diversity of *Rickettsia* species, which are classified into at least 4 groups: "ancestral" group (AG), typhus group (TG), transitional group (TRG), and spotted fever group (SFG) rickettsiae (Gillespie et al., 2007).

Many *Rickettsia* species cause diseases in humans and animals, following transmission by lice, fleas, ticks, or mites. Most of the recognized pathogenic *Rickettsia* species are classified into the SFG group, which includes the agents of spotted fever rickettsiosis that are transmitted by ticks to humans in different parts of the world (Parola et al., 2005b). During the past few decades, there has been an increasing number of reports of new *Rickettsia* species of unknown pathogenicity, mostly within ticks. In Colombia, the first cases of rickettsioses were reported in 1937 (Patino et al., 2006). More recently, 3 outbreaks of rickettsioses occurred in the country, in Necocli in 2006 (Antioquia) (Acosta et al., 2006), in the township of Los Cordobas (Córdoba) in 2007 (Hidalgo et al., 2011) and in Altos de Mulatos (Antioquia) in 2008 (Pacheco et al., 2008a). *Rickettsia rickettsii* was confirmed in all outbreaks, and until 2012, it was the only tick-associated rickettsia known to occur in Colombia. In 2012, a novel agent of unknown pathogenicity, *Candidatus* Rickettsia sp. strain Colombianensi, was reported in ticks (mostly *Amblyomma dissimile*) from Córdoba (Miranda et al., 2012).

R. bellii is the most widespread rickettsia in the Americas. It has been isolated from different species of ticks only in North America, namely from *Dermacentor variabilis*, *D. andersoni*, *D. occidentalis*, *D. albopictus*, *Haemaphysalis leporispalustris*, *Ornithodoros concanensis*, and *Argas cooleyi* collected in Montana, California, North Carolina, and Ohio (Philip et al., 1983). In South America, *R. bellii* has been detected and isolated from various tick species in some countries with different rates of infection (Labruna et al., 2004a, 2004b, 2007, 2011). Recently, serological evidence of infection by *R. bellii* was reported in animals (Pacheco et al., 2007); however, there is no evidence for human infection. The current phylogenetic classification of *R. bellii* is in the bellii group (BG), but this remains controversial (Weinert et al., 2009).

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The aim of this study was to provide molecular evidence of *Rickettsia* spp. of ticks collected from 2 sites of Cordoba.

Materials and methods

From May to June 2009, a total of 1069 *Amblyomma cajennense* ticks (783 males, 175 females, and 111 nymphs) were removed from 40 capybaras (*Hydrochoerus hydrochaeris*) in a rural area of Monteria, the capital city of the state of Córdoba (8°31′20″ N. 75°50′38″ W in north-western Colombia). All captured capybaras were infested by ticks. This area is characterized by tropical dry forest, with an average temperature of 28 °C.

Additionally, 458 *Amblyomma* sp. larvae and 20 *Amblyomma* sp. nymphs were collected in a rural locality of Los Cordobas, Cordoba (8°53′59″ N, 76°21′59″ W, northern coast of Colombia) by drag sampling on vegetation from January to March of 2009. The area of Los Cordobas, mostly used for agriculture and livestock, is a tropical dry forest with an average temperature >24 °C and 20–100 m above sea level.

Ticks were identified by taxonomic keys (Barros-Battesti et al., 2006). Specimens were grouped into 214 pools: 194 pools containing 2–5 *A. cajennense* individuals (adults or nymphs) from the same host, and 15 pools containing up to 30 *Amblyomma* sp. larvae, and 5 pools with 4 *Amblyomma* sp. nymphs. DNA from pools was extracted by using a QIAamp DNA Mini-Kit (QIAGEN, Valencia, CA, USA) and eluted in a final volume of 100 μl. Purified DNA was stored at −20 °C until used as a template for polymerase chain reaction (PCR). For initial screening of rickettsial infection, 5 μl of each pooled tick DNA template were used for real-time PCR (RT-PCR). RT-PCR was performed using a LightCycler rapid thermal cycler system (Roche Diagnostics, Mannheim, Germany). CS-5 and CS-6 primers were used, targeting a 147-bp fragment of the citrate synthase (*gltA*) gene (Labruna et al., 2004b). The hydrolysis probe assays contained a FastStart Taq DNA Polymerase (Roche) for hot-start PCR. For each reaction, both positive (*Rickettsia amblyommii* DNA) and negative (water) controls were included. An internal PCR control (Phage Lambda Genomic Control, designed for TIB MOLBIOL, LLC. Adelphia, NJ, USA) was used in all pool samples.

Positive samples were further analyzed using *gltA* (401 bp), *ompA* (631 bp), *ompB* (811 bp), and 16S ribosomal RNA (426 bp) gene, PCR assays, and sequencing (Labruna et al., 2004b; Marquez et al., 1998; Roux et al., 1996; Roux and Raoult, 2000). PCR assays were performed in automated MJ Research PTC-100TM thermal cyclers. A recombinant Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA) was used. PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and examined using an ultraviolet transilluminator (ImageQuant 100, Uppsala, Sweden). The PCR products were purified using a PureLink™ Quick Gel Extraction kit (Invitrogen) according to the manufacturer's instructions. Both strands of each gene fragment were directly sequenced; each sample was sequenced twice. Phylogenetic analyses were conducted with MEGA version 5 (Tamura et al., 2011). Partial DNA sequences obtained from the amplified PCR products (*gltA*, *ompA*, *ompB*, and 16S ribosomal RNA) were aligned with the corresponding sequences of other *Rickettsia* species available in GenBank using MEGA version 5. Phylogenetic distances between homologous sequences were calculated by using the Kimura's two-parameter model. For each analyzed gene a phylogram was constructed by the maximum likelihood method. Confidence values for individual branches of the resulting tree were determined by bootstrap analysis with 1000 replicates. For the *gltA*, a *R. bellii* sequence retrieved from GenBank (U59716) was designated as the outgroup (Roux et al., 1997); for the *ompA* analysis, *R. australis* was used as the outgroup (Stenos and Walker, 2000).

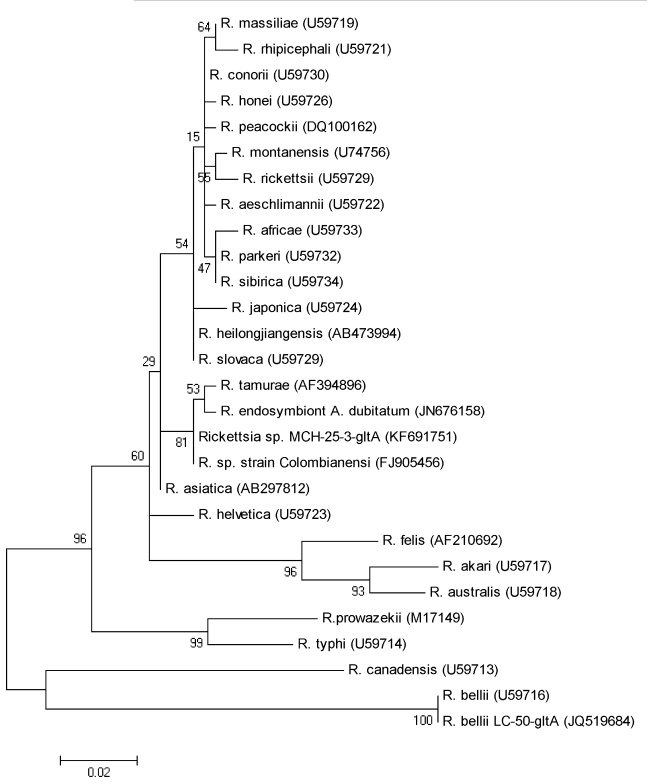

Fig. 1. Molecular phylogenetic analysis of a novel spotted fever *Rickettsia* sp. strain Colombianensi (MCH-25-3-*gltA*) from the tick *Amblyomma cajennense*. A total of 350 unambiguously aligned nucleotide sites of the rickettsial gene *gltA* were subjected to analysis by the maximum likelihood method. Bootstrap values for 1000 replicates are shown at the nodes. Bar, 0.02 substitution. The GenBank accession numbers of the sequences included in this analysis are shown in brackets.

Results

Rickettsial DNA was detected in 10 (4.6%) out of 214 pools of ticks by RT-PCR targeting the *gltA* gene. Overall, 5 (2.6%) pools from *A. cajennense* (obtained from 4 capybaras) from a rural area of Monteria, as well as 5 (33%) of free-living *Amblyomma* sp. larvae pools from Los Cordobas were positive. No pools of *Amblyomma* sp. nymphs showed rickettsial DNA by RT-PCR.

The 5 pools (LC-55, LC-51, LC-50, LC-44, and LC-41) of free-living larvae generated a PCR product only from the *gltA* gene; all of these pools were directly sequenced and the nucleotide sequences were 100% identical to each other. BLAST analysis of these partial sequences (350 bp) showed 100% identity to the sequence of *R. bellii* (GenBank EU826511, DQ865204, U59716, and DQ517288). The analysis of the partial sequence of *gltA* (LC-50-*gltA*) grouped in a distinct cluster (high bootstrap support 100%) with the sequence of *R. bellii* (GenBank U59716) (Fig. 1). The partial nucleotide sequence (350 bp) of the *gltA* gene has been deposited in the GenBank database under the accession number JQ519684.

Using conventional PCR, only one pool from *A. cajennense* (MCH-25-3) generated products from *gltA* (MCH-25-3-*gltA*), *ompA* (MCH-25-3-*ompA*), *ompB* (MCH-25-3-*ompB*), and 16S rRNA (MCH-25-3-16SrRNA) genes. The partial nucleotide sequences of these genes have been deposited in the GenBank database under the accession numbers KF691751, KF691749, KF691752, and KF691750, respectively. These fragments were directly sequenced, and the partial nucleotide sequences of these gene were 100% identical to nucleotide sequences of *gltA* (350 bp), *ompA* (556 bp) and

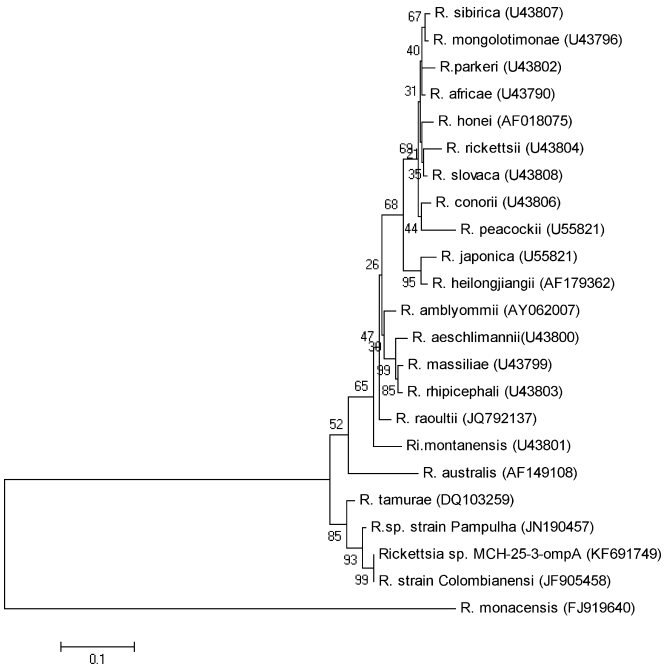

Fig. 2. Molecular phylogenetic analysis of a novel spotted fever *Rickettsia* sp. strain Colombianensi (MCH-25-3-*ompA*) from the tick *Amblyomma cajennense*. A total of 556 unambiguously aligned nucleotide sites of the rickettsial gene *ompA* was subjected to analysis by the maximum likelihood method. Bootstrap values for 1000 replicates are shown at the nodes. Bar, 0.1 substitution. The GenBank accession numbers of the sequences included in this analysis are shown in brackets.

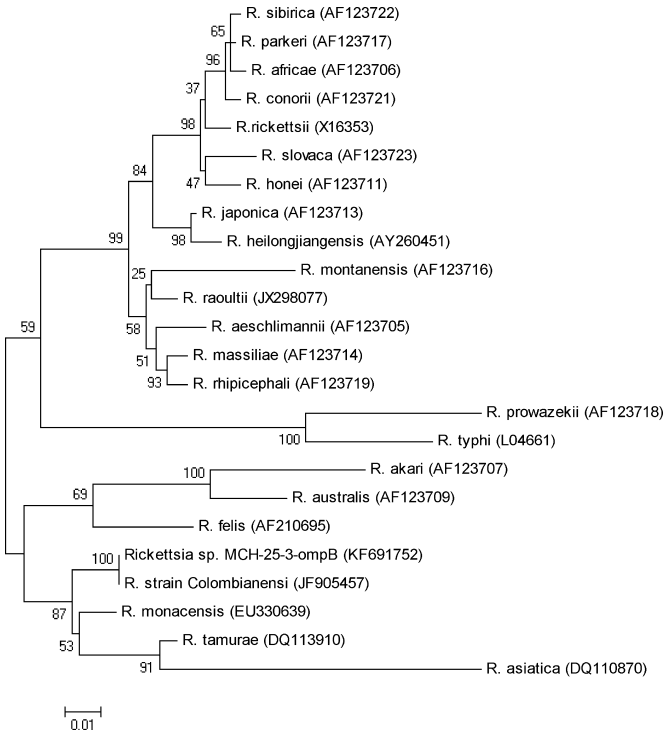

Fig. 3. Molecular phylogenetic analysis of a novel spotted fever *Rickettsia* sp. strain Colombianensi (MCH-25-3-*ompB*) from the tick *Amblyomma cajennense*. A total of 722 unambiguously aligned nucleotide sites of the rickettsial gene *ompB* was subjected to analysis by the maximum likelihood method. Bootstrap values for 1000 replicates are shown at the nodes. Bar, 0.01 substitution. The GenBank accession numbers of the sequences included in this analysis are shown in brackets.

ompB (722 bp) of a new proposed species *Candidatus* Rickettsia sp. strain Colombianensi (GenBank JF905456 for *gltA*, JF905458 for *ompA*, and JF905457 for *ompB*). This new *Rickettsia* showed partial *gltA* sequence 99.4% (348/350) identity with *Rickettsia tamurae* strain AT-1 (AF394896) and 99.1% (347/350) identity with *Rickettsia monacensis* (HM210740) and *Rickettsia asiatica* (AB297812), the closest validated species (Miranda et al., 2012). The partial sequence of the *ompA* gene was 96% (557/580) identical to the corresponding sequence of *R. tamurae* strain AT-1 (DQ103259) and 92.2% (535/580) to *R. monacensis* (FJ919650). The *ompB* gene nucleotide sequence showed 97.5% (689/707) genetic identity with the sequence of *R. monacensis* strain Irr/Munich and 97% (683/707) identity to the corresponding sequence of *R. tamurae*. For the partial MCH-25-3-16SrRNA gene sequence, 99.5% (380/382) identity with the sequence of *Rickettsia rhipicephali* (NR_025921) was found as the closest validated species.

Analysis inferred by the 4 rickettsial genes showed that *Candidatus* Rickettsia sp. strain Colombianensi belongs to the SFG group, since it grouped within a cluster that comprised the SFG *Rickettsia* species. Analysis of the *gltA* and *ompA* gene partial sequences showed that strain Colombianensi is in the same cluster as *R. tamurae*, which is supported by a high bootstrap value of 81% for *gltA* and a bootstrap value of 85% for the *ompA* gene (Figs. 1 and 2).

Notably, for *ompB*, strain Colombianensi also formed a lineage with *R. monacensis* and *R. tamurae* with a bootstrap value of 87% (Fig. 3). Analysis of a partial 16S rRNA gene sequence showed strain Colombianensi with several SFG species next to *R. rhipicephali*, but with a low bootstrap value of 46% (Fig. 4).

Discussion

Recent worldwide reports have renewed interest in assessing vectors for *Rickettsia*, due to the increasing number of emerging and reemerging diseases caused by different *Rickettsia* species (mostly SFG). New medical entomological studies have increased our understanding of rickettsial diseases in some countries, for example Brazil and Argentina. However, although in Colombia some sporadic outbreaks of rickettsial diseases occurred in recent years, research in that field is scarce. The present work is only the second medical-acarological study in the north and one of the first in the country investigating the presence of *Rickettsia* spp. in ticks.

This study evaluated rickettsial infection in ticks collected in Monteria and Los Cordobas (outbreak of RMSF in 2007; Hidalgo et al., 2011). Our results showed that of 214 pools evaluated (1547 ticks), 4.6% were positive for rickettsial DNA. Five pools containing free-living *Amblyomma* sp. larvae were positive only for the *gltA* gene; the sequences obtained from these 5 pools showed 100% identity to the sequence of *R. bellii*. The detection of *R. bellii* in this country confirms its extensive range in America. In North America, *R. bellii* was isolated from different families and genera of ticks; collected in Montana, California, North Carolina, and Ohio (Philip et al., 1983). *Rickettsia bellii* in South America was reported in Brazil and Argentina in different tick species with variable infection rates. In Brazil, in the state of Rondonia, Labruna et al. (2004a) found *R. bellii* in ticks of the species *Amblyomma ovale*, *A. scalpturatum*, *A. oblongoguttatum*, *A. rotundatum*, and *A. humerale*. In the same country, *R. bellii* was found in *A. dubitatum* (reported as *A. cooperi*) with a prevalence of 40% (Labruna et al., 2004b). In other areas of Brazil, Pinter and Labruna (2006) described *R. bellii* in *A. aureolatum* collected in Taiacupeba, a Brazilian spotted fever endemic area in the state of Sao Paulo. In Brazil, *R. bellii* was also found in *Ixodes loricatus* (Horta et al., 2006) and *Amblyomma nodosum* (Sabatini et al., 2010). In Argentina, in the Cordoba Province, Labruna et al. (2007) reported that 4.0% of *Amblyomma neumanni* were infected with *R. bellii*. In the same country, Tomassone et al. (2010), reported evidence of *R. bellii* infection in *A. tigrinum* ticks. No other reports of *R. bellii*-infected ticks have been published in South America (Labruna et al., 2011).

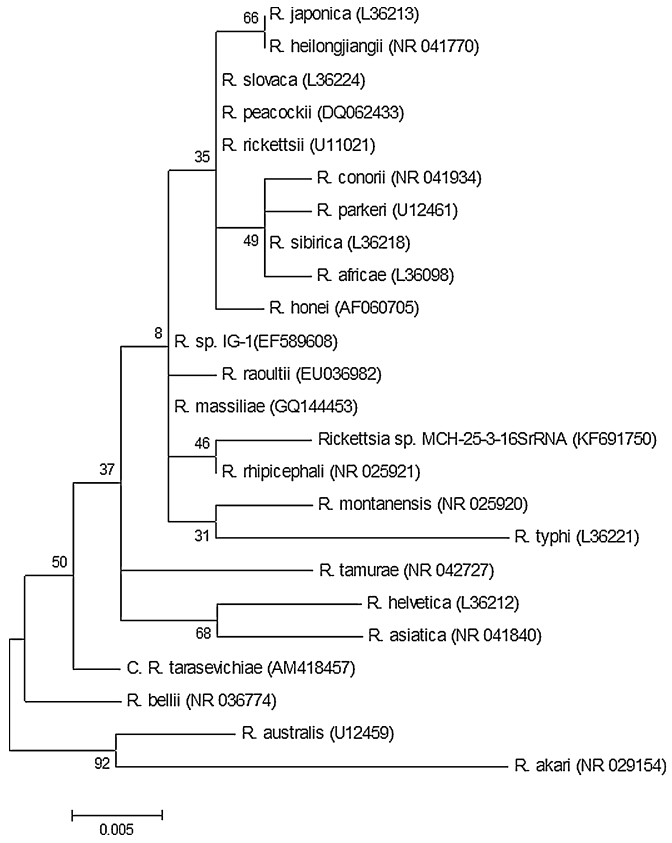

Fig. 4. Molecular phylogenetic analysis of a novel spotted fever *Rickettsia* sp. strain Colombianensi from the tick *Amblyomma cajennense*. A total of 360 unambiguously aligned nucleotide sites of the rickettsial gene 16S rRNA was subjected to analysis by the maximum likelihood method. Bootstrap values for 1000 replicates are shown at the nodes. Bar, 0.005 substitution. The GenBank accession numbers of the sequences included in this analysis are shown in brackets.

In the *Rickettsia* genus, *R. bellii* is the species infecting the widest variety of tick species (Pacheco et al., 2008b). The present study reports for the first time *R. bellii* in *Amblyomma* ticks from Colombia; however, the tick larvae were only identified at the genus level due to the lack of sufficient literature regarding the *Amblyomma* larval stage of the South American ixodid fauna. More studies are necessary to resolve this point.

R. bellii was found in the township of Los Cordobas, where outbreaks of *R. rickettsii* have been reported. Some authors observed antigenic cross-reactivity between *R. rickettsii* and *R. bellii* (Horta et al., 2004; Philip et al., 1983); this cross-reactivity could complicate seroprevalence studies using *R. rickettsii* as an antigen to determine the presence of antibodies against SFG rickettsiae.

On the other hand, the role of *R. bellii* as a human pathogen remains unknown, and the main ecological implication is that a less pathogenic *Rickettsia* species within a tick population could minimize the transmission of pathogenic *Rickettsia* species (Macaluso et al., 2002).

In *A. cajennense* ticks collected from capybaras in Monteria, we detected rickettsial DNA with 100% sequence identity to a possible new *Rickettsia* species recently found in *Amblyomma dissimile*, with the proposed name *Rickettsia* sp. strain Colombianensi (Miranda et al., 2012). Fournier et al. (2003), in a report of gene sequence-based criteria for identification of new *Rickettsia* species, proposed the following for a rickettsia to be classified as a new *Rickettsia* species: An isolate should not exhibit more than one of the following degrees of nucleotide similarity with the most homologous validated species: ≥99.8 and ≥99.9% for the *rrs* and *gltA* genes, respectively, and, if they can be amplified, ≥98.8, ≥99.2, and ≥99.3% for the *ompA* and *ompB* genes and gene D, respectively (Fournier et al., 2003). Although we reported partial sequences for 4 of these genes (*gltA*, *ompA*, *ompB*, and 16S rRNA) for this rickettsial DNA detected, our data support the hypothesis that the strain Colombianensi is a new species in the *Rickettsia* genus.

Rickettsia sp. strain Colombianensi is closely related to *R. tamurae*, a rickettsia species isolated from *Amblyomma testudinarium* ticks in Japan (Fournier et al., 2006). *Rickettsia tamurae* and strain Colombianensi are closely related to a recently described new genotype of rickettsia, designated as *Rickettsia* sp. strain Pampulha detected in *Amblyomma dubitatum* in Brazil (Almeida et al., 2011). The partial *ompA* sequence gene of strain Pampulha (JN190457) showed 96.2% identity (484/503) with *R. tamurae* (DQ103259), but this sequence has 97.6% identity (491/503) when compared with strain Colombianensi, indicating that both strains could be more related to each other than to *R. tamurae*. When compared with the partial sequence of *gltA*, strain Pampulha (JN190455), showed 99.8% identity (514/515) with *R. monacensis* (EU665236). In contrast, it was 99.0% identical (510/515) to *R. tamurae* (AF394896). However, the *gltA* gene of strain Pampulha could not be compared with our *gltA* sequence because different fragments of *gltA* were amplified. Nevertheless, further analysis of DNA sequences between strain Colombianensi and Pampulha is warranted.

The phylogenetic taxonomic status of strain Colombianensi as a new species is supported by high bootstrap values inferred from the genes *gltA*, *ompA*, and *ompB* that showed values of 81%, 85%, and 87%, respectively. The 16S rRNA gene has a low bootstrap value (46%) with its phylogenetic group. The low bootstrap value is due to very small differences in 16S rRNA genes in the SFG rickettsiae. Therefore, phylogenetic association inferred from this gene would preclude any conclusion about the taxonomic status of the strain Colombianensi. To obtain a precise phylogenetic analysis for rickettsia, it is necessary to use other criteria (*gltA*, *ompA*, *ompB*).

The presence of *R. rickettsii* has been demonstrated previously in the present study locations, (Hidalgo et al., 2011), but it was not evident by the methods used in this investigation. This has been reported in other endemic areas of *R. rickettsii* in America (Guedes et al., 2005; Pinter and Labruna, 2006), apparently because *R. rickettsii* has a low prevalence of ≤1% in different tick species.

Possible explanations for the low percentage of ticks infected with *R. rickettsii* were demonstrated experimentally by Niebylski et al. (1999), who showed highly lethal infection of *R. rickettsii* in *D. andersoni* ticks. Another possible explanation is antagonism, i.e., the infection of ticks with one rickettsia species precludes secondary infection with other rickettsiae (Macaluso et al., 2002).

The main important public health aspect of the present work is the fact that *A. cajennense* was found infected with *Candidatus* Rickettsia sp. strain Colombianensi in a different place from the previous report (Miranda et al., 2012). *Amblyomma cajennense* has been reported to be the most frequent human-biting tick in many areas of South America, including Colombia. This tick is also the most important vector of *R. rickettsii* in Central and South America (Guglielmone et al., 2006; Parola et al., 2005b). Thus, *A. cajennense* could allow the transmission of *Candidatus* Rickettsia sp. strain Colombianensi to humans, potentially leading to rickettsial disease.

A possible explanation for the infection of *A. cajennense* ticks with the strain Colombianensi in the study areas is that it is very common to find iguanas parasitized with *A. dissimile* ticks on their neck. It is also very common in the same areas to find domestic mammals parasitized with *A. cajennense*. This geographical overlap would allow immature stages of these ticks to cofeed on the same mammalian hosts where they would get infected with the strain Colombianensi.

Although the pathogencity of *Candidatus* Rickettsia sp. strain Colombianensi is unknown, other pathogenic rickettsiae, such as *Rickettsia parkeri*, *Rickettsia slovaca*, *R. aeschlimannii*, *R. massiliae*,

and *R. monacensis*, all SFG agents, were first reported to infect ticks, and only years later they were shown to cause human disease (Jado et al., 2007; Parola et al., 2005a). Some authors suggested that all *Rickettsia* have the potential to cause disease in vertebrates and claimed that opportunity for transmission to vertebrate hosts is the limiting factor in determining the probability of disease, but this hypothesis is controversial (Perlman et al., 2006).

Further studies are required to obtain cultured isolates of *Candidatus* Rickettsia sp. strain Colombianensi, which would make it easier to investigate it more closely and to confirm its taxonomic status as a new species. While strain Colombianensi is indeed a SFG rickettsia, its role as a human pathogen is unknown. If *Rickettsia* sp. strain Colombianensi is a pathogenic rickettsia, exposure to infected *A. cajennense* and *A. dissimile* ticks may pose a health risk (Miranda et al., 2012).

This is the first report of *R. bellii* in ticks in Colombia and the second report of *Rickettsia* sp. strain Colombianensi. Further studies are required to characterize their ecology and their pathogenic potential for humans.

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