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Detection of rickettsiae in fleas and ticks from areas of Costa Rica with history of spotted fever group rickettsioses

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ABSTRACT

Outbreaks of spotted fevers have been reported in Costa Rica since the 1950s, although vectors responsible for transmission to humans have not been directly identified. In this study, species of *Rickettsia* were detected in ectoparasites from Costa Rica, mostly from five study sites where cases of spotted fevers have been reported. Ticks and fleas were collected using drag cloths or directly from domestic and wild animals and pooled according to species, host, and location. Pools were analyzed initially by PCR to detect a fragment of *Rickettsia* spp. specific *gltA* gene, and those positive were confirmed by detection of *htrA* and/or *ompA* gene fragments. Partial sequences of the *gltA* gene were obtained, as well as at least one *htrA* and/or *ompA* partial sequence of each species. *Rickettsia* spp. were confirmed in 119 of 497 (23.9%) pools of ticks and fleas analyzed. *Rickettsia rickettsii* was identified in one nymph of *Amblyomma mixtum* and one nymph of *Amblyomma varium*. Other rickettsiae present were '*Candidatus* Rickettsia amblyommii' in *A. mixtum*, *Amblyomma ovale*, *Dermacentor nitens*, and *Rhipicephalus sanguineus* s. l.; *Rickettsia bellii* in *Amblyomma sabanerae*; *Rickettsia felis* in *Ctenocephalides felis*; and *Rickettsia* sp. similar to '*Candidatus* R. asemboensis' in *C. felis, Pulex simulans, A. ovale*, *Rhipicephalus microplus*, and *A. mixtum*. Results show the presence of rickettsiae in vectors that may be responsible for transmission to humans in Costa Rica, and evidence suggests exposure to rickettsial organisms in the human environment may be common. This is the first study to report *R. rickettsii* in *A. varium* and in *A. mixtum* in Costa Rica.

Key words: *Rickettsia*; Ixodida; Siphonaptera; Ectoparasite; Tick-borne disease; Central America

Introduction

In Latin America, the only rickettsial diseases recognized during the 20th century were Rocky Mountain spotted fever (RMSF), flea-borne typhus, and epidemic typhus caused by *Rickettsia rickettsii, Rickettsia typhi,* and *Rickettsia prowazekii,* respectively (Labruna et al., 2011). The description of new species of these intracellular ectoparasite-borne bacteria has increased in the past 25 years, and emerging human pathogens have been documented worldwide (Parola et al., 2005, 2013). There are now at least 10 more species of *Rickettsia* reported in Latin America, including known human pathogens (*Rickettsia akari*, *Rickettsia felis*, *Rickettsia parkeri*, *Rickettsia massiliae, Rickettsia africae*) and previously undescribed species and genotypes (Pacheco et al., 2007, 2011; Labruna et al., 2011; Miranda et al., 2012; Spolidorio et al., 2012; Troyo et al., 2014). Although infection by some of the rickettsiae present in the region may be severe or have a high fatality rate if untreated, others such as *Rickettsia bellii*, *Rickettsia rhipicephali*, and '*Candidatus* Rickettsia amblyommii' are considered nonpathogenic or have not been associated conclusively with disease in humans (Mediannikov et al., 2007; Labruna et al., 2011; Parola et al., 2013).

In Central America, the first human cases of spotted fever were documented in Panama and Costa Rica during the 1950s (de Rodaniche and Rodaniche, 1950; Calero et al., 1952; Fuentes, 1979; Hun-Opfer, 2008). In other countries of Central America, reports of spotted fever group (SFG) rickettsiosis are more recent and include diagnosis in a traveler returning from Honduras (Chen and Wilson, 2009), and a possible outbreak that was identified in Guatemala in 2007 with at least one confirmed case (Eremeeva et al., 2012). In El Salvador and Nicaragua, cases of spotted fevers have not been officially confirmed, but there is serological evidence of infection in both countries (Peacock et al., 1971; WHO, 1993). In the

case of Belize, there are no reports of human infection with rickettsiae, although the pathogenic *Rickettsia* sp. strain Atlantic rainforest was recently reported infecting ticks in that country (Lopes et al., 2016).

The ecology of rickettsial diseases has been poorly investigated in most countries of Central America (Labruna et al., 2011), despite the evidence of possible transmission in most of the region and recurrent case reports and fatalities in Costa Rica and Panama (Estripeaut et al., 2007; Tribaldos et al., 2011; Arguello et al., 2012; De Lucas et al., 2013; Hun, 2013). In Costa Rica, *R. rickettsii* was identified and confirmed as the species responsible for human cases (Hun et al., 2008), although the most complete ecological studies of RMSF date from the 1980s (Fuentes et al., 1985; Fuentes, 1986). In these investigations, *R. rickettsii* was detected and isolated only on one occasion from an invertebrate host, the rabbit tick *Haemaphysalis leporispalustris* (Fuentes et al., 1985; Fuentes, 1986).

Considering that information concerning vectors and reservoirs of rickettsiae in Costa Rica is limited, a project was initiated in 2008 to detect and identify species of rickettsia in ticks and fleas that may present a risk of contact and infection for the human population. Preliminary results of this study documented the presence and isolation of '*Candidatus* R. amblyommii' and *R. felis* for the first time in Costa Rica (Hun et al., 2011; Troyo et al., 2012a). This paper adds substantial evidence to complete the detection and identification of rickettsiae in different ectoparasite species, collected predominantly from domestic and peridomestic animals in areas of Costa Rica where cases of spotted fevers have historically been diagnosed or are suspected.

Materials and methods

Study sites

The main collection sites have been described previously (Troyo et al., 2012b). Sites are located to the North and East (Caribbean) regions of Costa Rica and have reported cases of spotted fevers in the past (Campbell et al., 1978; Fuentes, 1979; Hun et al., 1991; Hun-Opfer, 2008). Specifically, 5 sites were selected in 7 districts: 1) Turrialba (9°54' N, 83°41' W; elevation: 650 m.a.s.l.), 2) La Virgen (10°23' N, 84°08' W; elevation: 190 m.a.s.l.), 3) Limón (9°59' N, 83°02' W; elevation: 5 m.a.s.l.), 4) Cahuita (9°44' N, 82°50' W; elevation: 5 m.a.s.l.), 5) Guápiles (10°13' N, 83°47' W; elevation: 260 m.a.s.l.), Jiménez (10°12' N, 83°44' W; elevation: 230 m.a.s.l.), and Guácimo (10°12' N, 83°41' W; elevation: 110 m.a.s.l.). The urban centers of Guápiles, Jiménez, and Guácimo (GP/J/GC) are less than 20 km from each other and were therefore considered to be the same study area (one site). These regions are characterized by continuous wet and warm conditions without distinct seasonality: annual rainfall is 2,500—3,500 mm, and mean minimum and maximum temperatures are approximately 20 °C and 30 °C, respectively (CRRH, 2008).

The Vector Research Laboratory (Laboratorio de Investigación en Vectores, LIVE) of the University of Costa Rica (UCR), provides the general public with a service of counseling and identification of medically important arthropods. Taking advantage of this service, additional tick and flea samples that were received by the laboratory from other areas of the country were included in the analyses.

Collection and identification of ectoparasites

Ectoparasites analyzed were collected or received at the LIVE, UCR, between July 2008 and March 2013. At study sites, a non-probabilistic approach was used to target households, farms, and other private properties for collection of specimens. These locations

were set preferably in rural environments and in proximity to forested areas. Domestic animals were identified, and 20 to 25 live animal traps (homemade wooden box, Tomahawk® and/or Havaheart® traps) were placed one day and collected the next day (after 20-24 hours) to capture opossums, rodents, and other small mammals in the surrounding areas. Traps were placed, in most cases, for 2-3 consecutive days and at least once every year at each site during 2008-2012. Small animals trapped were measured, photographed, and liberated after inspection and collection of ticks and fleas. Identification of animals was made using general descriptions (Reid, 2000), which were confirmed by an expert mammologist. All methods for trapping and manipulating animals, as well as collecting ectoparasites in this study followed the "Regulations about access to the biodiversity in teaching, social action, and research activities of the University of Costa Rica" (projects A8-127 and B1-041), the "Law of Biodiversity 7788" of Costa Rica, and were approved by University of Costa Rica's Institutional Committee for the Use and Care of Laboratory Animals (CICUA-35-10).

Fleas, ticks, and other ectoparasites were collected from their animal hosts for a period no longer than 1 man-hour per animal using combs and forceps (for example, collection would not take more than 30 minutes if two people were collecting from an animal, or no more than 15 minutes if there were 4 people). Ticks and fleas from other wild animals (reptiles, toads, sloths, raccoons) were collected during independent rescue and/or veterinary activities and were brought to the LIVE by third parties for identification and analysis. Specimens from different host species, collection sites, and specific locations (household, farm, property) were placed separately in dry glass vials (ticks), or vials with water or 70% ethanol (fleas and other ectoparasites). Specimens were kept live and at 4 ºC (< 7 days) or frozen at -20 ºC until processing for identification.

When required for species identification or confirmation, some of the specimens were cleared in lactophenol and mounted in Hoyer's medium. Ticks (Ixodida), and fleas

(Siphonaptera) were selected and identified by observing morphological characters and using identification keys (Hopkins and Rothschild, 1953; Smit, 1958; Barros-Battesti et al., 2006; Vargas, 2006). Ticks belonging to the *Amblyomma cajennense* species group were considered to be *Amblyomma mixtum*, which is considered to be the only representative species of this group in Central America (Nava et al., 2014). In addition, those identified as *Rhipicephalus sanguineus* were designated as "sensu lato" (s. l.), since the presence of 2 species of this group has been established in Latin America (the tropical lineage is the one present in Central America) (Nava et al., 2015).

Depending on the size and total number collected, specimens were grouped into pools containing usually 1 to 5 adult ticks (up to 10), 1 to 20 tick nymphs and/or larvae (up to 50), and 1 to 10 fleas from the same ectoparasite species, vertebrate host species, and location. Once prepared, all pools were conserved at –20 °C previously to PCR analyses.

In two pools that were of special interest, molecular confirmation of the tick species was performed by amplifying and sequencing a fragment of the mitochondrial 16S ribosomal RNA gene using primers 16S+1 and 16S-1 (460 bp product) (Black and Piesman, 1994; Mangold et al., 1998). DNA extraction, sequencing, editing, and analyses were performed in the same manner described in the section below.

Detection and identification of Rickettsia *spp.*

Ticks and fleas were washed 3 times in 0.1% iodine and 70% ethanol solution and 3 times in sterile distilled water prior to DNA extraction, to reduce external bacteria and other possible contamination. Genomic DNA was extracted from ectoparasite pools using QIAquick® Gel Extraction Kit (Qiagen) (2008-2010) or NucleoSpin® Tissue kit (Macherey-Nagel) (2011-2013), following the manufacturers' instructions. DNA from each pool was

analyzed by end-point polymerase chain reaction (PCR) for detection of *Rickettsia*-specific portions of the citrate synthase (*gltA*), serine protease (*htrA*), and outer membrane protein A (*ompA*) genes, as described previously (Troyo et al., 2012a). Briefly, a first PCR to detect the *Rickettsia* spp. *gltA* was performed in all pools using primers CS-78 and CS-323, which amplify a 401 bp product of a conserved region (Labruna et al., 2004). To confirm positivity and recognize SFG rickettsiae, a subsequent detection of *htrA* and *ompA* fragments was performed in pools positive for the *gltA* using nested and semi-nested PCRs. Primers used for detection of *htrA* were R17-122 and R17-500 (380 bp fragment specific for *Rickettsia* spp.), and a subsequent nested step with TZ15 and TZ16 (SFG-specific fragment of 247 bp) or RP2 and RPID (Typhus Group-specific fragment of 286 bp) (Anderson and Tzianabos, 1989; Tzianabos et al., 1989; Massung et al., 2001). For *ompA*, primers used were Rr190-70 and Rr190-701, as well as Rr190-70 and Rr190-602 for a semi-nested PCR (632 and 532 bp fragments, respectively, for SFG *Rickettsia*) (Regnery et al., 1991; Roux et al., 1996). Pools were considered positive for *Rickettsia* spp. when the expected fragments for at least two of the three genes analyzed were detected by PCR. In addition, a minimum infection rate (MIR) was calculated for each species of ectoparasite [(number of positive pools/total number of ticks or fleas tested) × 100].

To identify *Rickettsia* species, amplicons of pools that tested positive for *gltA* were sequenced. PCR products were purified using BigDye® XTerminator™ Purification Kit, sequenced with BigDye™ Terminator V 3.1, and visualized in the Genetic Analyzer 3130 (Applied Biosystems/HITACHI). Positive amplicons obtained in 2012 and 2013 were purified with Exonuclease I and FastAP (Thermo Fisher Scientific Inc.) and sequenced in Macrogen, Inc. (Seoul, South Korea). All sequences were edited and assembled using DNA Baser (DNA Sequence Assembler v4, 2013, Heracle BioSoft, www.DnaBaser.com), and compared by BLAST against the NCBI database to search for similar sequences. In addition, *ompA*

fragments (primers Rr190-70 and Rr190-701) or *ompB* fragments (primers 120-M59f/120-807; 856 bp product) (Roux and Raoult, 2000) were sequenced and analyzed in a similar manner to confirm the identity in at least one pool of each species of *Rickettsia* that was detected.

Results

A total 4 588 ticks and fleas were obtained from different sources: cattle, horses, domestic dogs, cats, humans, rats, opossums, wood turtles, snakes, toads, sloths, raccoons, and vegetation/environment (Table 1). Ten species of ticks and 3 species of fleas were identified. In addition, several larvae and nymphs were only identified morphologically as *Amblyomma* spp.

Overall, 497 pools of ectoparasites were analyzed by PCR. The distribution of pools containing DNA of *Rickettsia* spp. and minimum infection rates (MIR), according to ectoparasite species and study site, are presented in Table 2. Detection of at least two of the three *Rickettsia* spp. gene fragments was possible in 119 (23.9%) pools. A high prevalence of *Rickettsia* spp. was observed in *Amblyomma* pools, especially in *A. mixtum* from horses (67.7% of *A. mixtum* pools; MIR: 9.9%), *Amblyomma ovale* from dogs (15.4% of *A. ovale* pools; MIR: 9.3%), and *Amblyomma sabanerae* from wood turtles (66.7% of *A. sabanerae* pools; MIR: 11.1%). However, only 3 pools of *A. sabanerae* were analyzed, and most of *A. mixtum* were from the Cahuita site. At all study sites, *Rickettsia* DNA was also frequent in pools of *Ctenocephalides felis* (44.9% of *C. felis* pools; MIR: 7.2%), most of which were collected from dogs. In contrast, rickettsiae were infrequent in pools of other common ectoparasites of the areas studied, including *Rhipicephalus microplus* (6.6% of *R. microplus* pools; MIR: 0.4%), *Rhipicephalus sanguineus* s. l. (1.3% of *R. sanguineus* s. l. pools; MIR: 0.1%), and *Pulex simulans* (7.1% of *P. simulans* pools, MIR: 1.0%) (Table 2).

Sequencing of the *Rickettsia* spp. *gltA* fragment and species identification with 99.6% to 100% DNA sequence similarity was possible for 100 (84%) of the positive pools (Table 3). After several attempts, it was not possible to obtain a good quality sequence for analysis from the remaining 19 PCR-positive pools (quality value scores less than 21, noise, overlapping peaks, etc.). According to BLAST search results, sequences of *gltA* fragments corresponding to *R. rickettsii* were detected in only two pools of ticks (Table 3). In both cases, specimens were collected and brought to the laboratory by a third party. Identification of *R. rickettsii* was further confirmed by re-analyzing the sample and sequencing the *gltA* and *ompA* amplicons in two different laboratories (Universidad de Costa Rica and Universidade de São Paulo). The same *gltA* results were obtained and the fragments showed 100% (349/349 for *gltA*, and 534/534 for *ompA*) sequence similarity with *R. rickettsii* (CP000848). Ticks in one of the pools were identified morphologically as *A. cajennense* s. l., although the information of specific location and host was unavailable. An analysis of the mitochondrial 16S ribosomal RNA gene from this tick determined that the highest partial sequence homology (99.3%; 400/403) was with a sequence of *A. mixtum* form Texas, USA (Accession No. KM458242) (Medlin et al., 2015). The second sample that was positive for *R. rickettsii* was a single nymph of *Amblyomma* collected near the metropolitan area of San José. It was received dead and in very poor condition for morphological identification; it was collected while attached to the same person who brought it (no symptoms of disease were reported). In this case, the 16S ribosomal RNA gene fragment showed 100% (404/404) sequence similarity with *Amblyomma varium* also from Costa Rica (Accession No. KF702341) (Ogrzewalska et al., 2015).

'*Candidatus* R. amblyommii' was the most common species of *Rickettsia* in ticks, according to similarity to several partial sequences available in GenBank, including those with accession numbers HM582435, DQ517290, and CP003334 (Table 3). This *Rickettsia* was detected in *A. mixtum*, *A. ovale*, *D. nitens*, and *R. sanguineus* s. l. In addition, *gltA* partial

sequences corresponding to *Rickettsia bellii* (CP000087) were present in *A. sabanerae* tick pools from wood turtles (Table 3).

Rickettsiae similar to '*Candidatus* Rickettsia asemboensis' (KJ569090) were identified in pools of *C. felis*, as was communicated preliminarily as *Rickettsia* sp. RF2125 (Troyo et al., 2012a). This *Rickettsia* was also detected in *P. simulans* and *A. ovale* collected from dogs, and in *R. microplus* from cows (Table 3).

As was done for *R. rickettsii*, the presence of '*Candidatus* R. amblyommii', *R. felis*, and '*Candidatus* R. asemboensis' in several samples was further confirmed by sequencing the *ompA* or *ompB* gene fragment, which included at least one sample corresponding to each of these rickettsiae. In all pools analyzed, *ompA* and *ompB* partial sequences obtained had 99.8-100% similarity with the corresponding species of *Rickettsia* that had been identified using the *gltA* fragment. Representative sequences of each *Rickettsia* were deposited in GenBank with accession numbers: KX544813 and KX544816 for *R. rickettsii*; KX544804 for *R. bellii*; KX544805, KX544806, KX544812, and KX544815 for '*Candidatus* R. amblyommii'; KX544809 and KX544814 for *R. felis,* and KX544807, KX544808, KX544810, KX544811, and KX544817 for '*Candidatus* R. asemboensis'. Accession numbers of the mitochondrial 16S ribosomal RNA gene fragment sequences of *A. mixtum* and *A. varium* were deposited as KX544819 and KX544818, respectively.

Discussion

Previous studies in Costa Rica reported the presence of *R. rickettsii* in rabbit ticks, *H. leporispalustris*, during the 1980s (Fuentes et al., 1985); however, no other rickettsiae species or confirmed tick vectors of spotted fevers had been documented in the country before the beginning of this project in 2008. In this study, *R. rickettsii* was detected in ticks of the genus

Amblyomma. Ticks from the *A. cajennense* complex or identified as *A. mixtum*, have been implicated as possible vectors of *R. rickettsii* to humans in Panama (de Rodaniche, 1953; Bermúdez et al., 2016); some of the other species of this complex are also vectors of *R. rickettsii* in South America (Krawczak et al., 2014; Faccini-Martínez et al., 2015). Although most of the *A. mixtum* ticks analyzed in the present study were collected from horses in Cahuita, some of the specimens were obtained from humans, as this species complex commonly bites humans (Guglielmone et al., 2006). Unlike *A. mixtum*, *A. varium* has not been implicated in transmission of pathogenic rickettsiae and only *R. bellii* and '*Candidatus* R. amblyommii' have been detected in this tick species (Ogrzewalska et al., 2012; Lugarini et al., 2015; Ogrzewalska and Pinter, 2016). Adults of *A. varium* are ectoparasites of the mammal families Bradypodidae and Megalonychidae (Xenarthra), and vertebrate hosts of immature stages can include birds but have not been well characterized (Ogrzewalska and Pinter, 2016). Although confirmation is still required, a study in the State of Pará, Brazil, reported numerous immature and adult stages of *A. varium* parasitizing humans (Serra-Freire, 2010), as was the *R. rickettsii*-infected nymph analyzed in the present study. However, there are no other records of human parasitism by *A. varium* (Guglielmone et al., 2014). In Costa Rica, this tick species and their vertebrate hosts are common in endemic areas and were documented at sites that were investigated following reports of RMSF cases, although no rickettsiae were obtained from them at that time (Fuentes, 1986; Hun et al., 1991). Therefore, the role of adult and immature stages of *A. mixtum* and other *Amblyomma* spp. of wild animals, including *A. varium,* as vectors of *R. rickettsii* should be evaluated further.

In contrast with the low infection rate of *R. rickettsii*, infection rate of *A. mixtum* with '*Candidatus* R. amblyommii' was high, which may be expected considering that it is a common finding in other countries of the region, including Honduras and Panamá (Novakova et al., 2015; Bermúdez et al., 2016). The infection rate in *A. ovale*, *D. nitens*, and *R.*

sanguineus s. l. was lower, but confirms previous reports of '*Candidatus* R. amblyommii' in these tick species (Bermúdez et al., 2009, 2011). The presence of '*Candidatus* R. amblyommii' in ticks that are known to bite humans, including *A. mixtum* and *A. ovale* (Guglielmone et al., 2006), indicates that people may be at risk of exposure to this *Rickettsia*. Even though '*Candidatus* R. amblyommii' is not considered a human pathogen and has only been associated indirectly with mild disease (Apperson et al., 2008), experimental infection has produced pathology in guinea pigs and should be studied further in individuals with specific health conditions such as immunosuppression (Rivas et al., 2015). In addition, exposure may induce a partial protective immunity to *R. rickettsii*, as has been suggested in experimental studies (Blanton et al., 2014; Rivas et al., 2015), which could modulate disease epidemiology and severity in endemic areas of the region, such as Cahuita.

Of note, *A. ovale* specimens analyzed in this study did not contain *Rickettsia* sp. strain Atlantic rainforest, which is a known human pathogen that has been detected in this tick species in Central America (Belize) and South America (Brazil and Colombia) (Barbieri et al., 2014; Londoño et al., 2014; Lopes et al., 2016). More analyses of *A. ovale* ticks are required, including specimens from wild animals, in order to determine if this pathogenic *Rickettsia* is present in Costa Rica.

The infection rate of *Rickettsia bellii* in turtle ticks (*A. sabanerae*) was high in this study, although only 3 tick pools were analyzed. This species of *Rickettsia* was reported recently in *A. sabanerae* larvae on birds from Costa Rica (Ogrzewalska et al., 2015), and it was reported previously in the same tick species from El Salvador (Barbieri et al., 2012). Experimental studies indicate that *R. bellii* can infect guinea pigs and opossums without apparent disease (Horta et al., 2010), and exposure to *R. bellii* has been documented by serology in dogs (Silva Fortes et al., 2010). Although the existence of amplifying vertebrate species has not been excluded, the high prevalence of *R. bellii* in *A. sabanerae* in the region is

probably the result of vertical transmission, which has been demonstrated as a very efficient transmission route in this *Rickettsia* (Horta et al., 2006).

In fleas, *R. felis* was only detected in *C. felis* from San José and not in the specific study sites, where only rickettsiae similar to '*Candidatus* R. asemboensis' were identified. This *Rickettsia* was first reported as *Rickettsia* sp. genotype RF2125 from the Thailand-Myanmar border (Parola et al., 2003), but it has been more recently referred to as '*Candidatus* R. asemboensis', following the description of a similar *Rickettsia* by Jiang et al. (2013) in Asembo, Kenya. Studies have shown that DNA sequences of the *gltA* and other genes from both rickettsiae are very similar; hence they are probably the same species (Jiang et al., 2013; Oteo et al., 2014; Faccini-Martínez et al., 2016). At study sites, infection rate with this *Rickettsia* was much higher in pools of *C. felis*, but they were also detected in *P. simulans*, which commonly co-infest dogs in these areas (Troyo et al., 2012b). The lower infection rate in *P. simulans*, despite a large number of flea pools analyzed from the same vertebrate hosts, agrees with other studies that show absence of rickettsiae in *Pulex* fleas and may indicate alternative and less-frequent infection routes, such as co-feeding (Oteo et al., 2014; Brown et al., 2015). In this regard, there are few reports of DNA of *R. felis* or similar rickettsiae in blood of dogs, and this may be an infrequent finding in the region (Wei et al., 2014). In addition, there may be specific bacteria-flea-dog interactions that limit infection and/or vertical transmission in populations of *P. simulans*. An opposite effect has been reported for other bacteria like *Bartonella* spp., where infection rates in *P. simulans* are higher (fleas probably acquire bacteria from a bacteremic dog) (Rojas et al., 2015). Even though infection rates were lower than in *C. felis,* '*Candidatus* R. asemboensis' was also detected in ticks including *A. ovale* and *R. microplus*, which can also infest dogs and where they may come in close contact with infected fleas. Moreover, it is important to note that positive PCR results may indicate

contamination with infected flea feces in specimens found on the same dog, or even the presence of bacteria in the animal's blood within the ectoparasite.

Although it is possible that some of the pools may have had more than one species of *Rickettsia* present (the most abundant is the one evidenced after sequencing), results from this study confirm the presence of 5 species of rickettsiae in ticks and fleas in Costa Rica, many of which are common on domestic animals and may pose a threat to human health (*R. rickettsii* and *R. felis*) or may impact the epidemiology of SFG rickettsioses (i.e. '*Candidatus* R. amblyommii'). Results indicate that rickettsiae are present in domestic and peridomestic areas, which provide an ecological context that may be suitable for their establishment and contact with humans. Therefore, awareness and recognition of rickettsial transmission in the region is necessary for adequate prevention and treatment, especially in areas where other vector-borne diseases that may challenge the differential diagnosis are common, such as dengue, chikungunya, Zika, and ehrlichiosis.

This is the first report of *R. rickettsii* in molecularly confirmed *A. mixtum* in Costa Rica and the first report of *R. rickettsii* in *A. varium*. Other new associations between rickettsiae and ectoparasite species include '*Candidatus* R. asemboensis' in *R. microplus*, *A. ovale*, and *P. simulans*. In addition, the presence of *R. bellii* and *R. rickettsii* in ticks that usually parasitize wild animals suggests that these species may be important in maintaining enzootic transmission cycles of these rickettsiae, which can potentially reach humans and/or domestic animals through infected immature stages. In addition to rabbits (Fuentes, 1986), other wild animal reservoirs or amplifying hosts of *R. rickettsii* have not been identified in endemic areas of Central America. Hence, the role of wild animals and their ticks in the transmission cycles of rickettsiae should be evaluated more thoroughly in the region.

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Table 1

Ticks and fleas collected at sites in Costa Rica and total pools analyzed for detection of *Rickettsia* spp. citrate synthase gene (*gltA*).

Ectoparasite	Vertebrate host/source	No. collected	Stages[†]	Pools analyzed
Amblyomma mixtum	Horse, human, vegetation/environment	243	A,N	31
Amblyomma dissimile	*Boa constrictor,* unidentified snake	15	A	2
Amblyomma geayi	Sloth (*Choloepus hoffmanni*)	1	A	1
Amblyomma ovale	Dog, cat, human	45	A	26
Amblyomma rotundatum	Toad (*Rhinella* sp.)	1	A	1
Amblyomma sabanerae	Wood turtle (*Rhinoclemmys funerea*)	20	A	3
Amblyomma sp.*	Vegetation, horse, dog, human, cow, opossum (*Philander opossum*, *Didelphis marsupialis*), spiny rat (*Proechimys semispinosus*)	233	N,L	19
Dermacentor nitens	Horse	881	A,N,L	39
Ixodes boliviensis	Dog	3	A	2
Rhipicephalus microplus	Cow, dog, horse, vegetation	804	A,N,L	45
Rhipicephalus sanguineus s. l.	Dog, cat, vegetation/environment	678	A,N,L	75
Adoratospylla intermedia copha	Opossum (*D. marsupialis, P. opossum*)	12	A	2
Ctenocephalides felis	Dog, cat, cow, raccoon, vegetation/environment	1 051	A	167
Pulex simulans	Dog	601	A	84
All species	All hosts/sources	4 588		497

*One *Amblyomma* sp. nymph from a human was later identified molecularly as *A. varium*.
[†] A: adults, N: nymphs, L: larvae.

Table 2

Minimum infection rate (MIR) and proportion of pools positive for *Rickettsia* spp. according to ectoparasite species and study site.

Ectoparasite	***Rickettsia* spp. positive pools per site**						***Rickettsia* spp. positive pools**	**MIR (%)**
	Cahuita	**Guápiles/ Jiménez/ Guácimo**	**La Virgen**	**Limón**	**Turrialba**	**Other sites**		
Amblyomma mixtum	12/22	-	-	-	1/1	8/8	**21/31 (67.7%)**	**9.9**
Amblyomma dissimile	-	-	-	-	-	0/2	**0/2 (0%)**	**0**
Amblyomma geayi	-	-	0/1	-	-	-	**0/1 (0%)**	**0**
Amblyomma ovale	3/10	0/9	-	-	0/1	1/6	**4/26 (15.4%)**	**9.3**
Amblyomma rotundatum	-	-	-	-	0/1	-	**0/1 (0%)**	**0**
Amblyomma sabanerae	-	-	-	-	-	2/3	**2/3 (66.7%)**	**11.1**
Amblyomma sp.*	0/4	0/4	0/1	0/6	-	3/4	**3/19 (15.8%)**	**1.4**
Dermacentor nitens	2/12	0/8	1/7	0/3	1/6	0/3	**4/39 (10.3%)**	**0.5**
Ixodes boliviensis	-	-	-	-	-	0/2	**0/2 (0%)**	**0**
Rhipicephalus microplus	0/2	0/12	2/9	0/1	1/11	0/10	**3/45 (6.6%)**	**0.4**
Rhipicephalus sanguineus s. l.	0/6	1/39	0/2	0/3	0/5	0/20	**1/75 (1.3%)**	**0.1**
Adoratopsylla intermedia copha	-	-	-	-	0/2	-	**0/2 (0%)**	**0**
Ctenocephalides felis	9/33	27/65	3/14	18/30	14/15	4/10	**75/167 (44.9%)**	**7.2**
Pulex simulans	2/20	1/26	1/8	1/11	1/12	0/5	**6/84 (7.1%)**	**1.0**

*One PCR-positive nymph was identified molecularly as *A. varium*.

Table 3

Rickettsia spp. identified in pools of ticks and fleas collected in Costa Rica, according to partial sequence homologies of the *gltA* amplicons.

Ectoparasite	No. sequences obtained	Similarity (%)	Length (bp)	*Rickettsia* sp. [GenBank accession number]	Vertebrate host/source
*Amblyomma mixtum**	18	99.6-100	293-349	'*Candidatus* R. amblyommii' [KM652483, CP003334]	Horses, cows, human, vegetation/environment
	1	100	349	*R. rickettsii* [CP000848]	Unknown
Amblyomma ovale	3	100	349	'*Candidatus* R. asemboensis' [KJ569090]	Dogs
	1	100	349	'*Candidatus* R. amblyommii' [KM652483]	Dog
Amblyomma sabanerae	2	100	342, 349	*R. bellii* [CP000087]	Wood turtle (*Rhinoclemmys funerea*)
*Amblyomma varium**	1	100	349	*R. rickettsii* [CP000848]	Human
Amblyomma sp.	1	100	349	'*Candidatus* R. amblyommii' [KM652483]	Horse
Dermacentor nitens	2	100	332, 349	'*Candidatus* R. amblyommii' [CP003334]	Horses
Rhipicephalus microplus	2	100	300, 349	'*Candidatus* R. asemboensis' [KJ569090]	Cows
Rhipicephalus sanguineus s. l.	1	100	349	'*Candidatus* R. amblyommii' [CP003334]	Dog
Ctenocephalides felis	60	99.6-100	235-349	'*Candidatus* R. asemboensis' [KJ569090]	Dogs, cats
	3	100	349	*R. felis* [CP000053]	Dogs
Pulex simulans	5	99.6-100	235-349	'*Candidatus* R. asemboensis' [KJ569090]	Dogs

* Tick identification was confirmed in pools containing *R. rickettsii* by molecular analysis of the mitochondrial 16S ribosomal RNA gene.