

Original Papers

Questing *Amblyomma mixtum* and *Haemaphysalis juxtakochi* (Acari: Ixodidae) Infected with *Candidatus* “*Rickettsia amblyommii*” from the Natural Environment in Panama Canal Basin, Panama

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Abstract: This work emphasizes the detection of *Candidatus* “*Rickettsia amblyommii*” in questing *Haemaphysalis juxtakochi* and *Amblyomma mixtum*. From February 2009 to December 2012, questing ticks were collected from the vegetation and leaf-litter of four protected forests and two grassy areas around the Panama Canal basin. DNA was extracted from *Amblyomma mixtum*, *Amblyomma naponense*, *Amblyomma oblongoguttatum*, *Amblyomma pecarium*, *Amblyomma tapirellum*, *Haemaphysalis juxtakochi*, and unidentified immature *Amblyomma*. Specific primers of citrate synthase gene *gltA* were used to detect and identify the rickettsiae. Amplicons with the expected band size were purified and sequenced. DNA of *C. “R. amblyommii”* was found in *A. mixtum*, *H. juxtakochi* and *Amblyomma* immatures. To our knowledge, these findings represent the first report of *C. “R. amblyommii”* in free-living ticks in the wilderness of Central America.

Key words: *Candidatus* “*Rickettsia amblyommii*”, Ixodidae, questing ticks, Panama Canal Basin

INTRODUCTION

The causative agents of tick-borne rickettsiosis (TBR) include 16 *Rickettsia* species with great relevance in worldwide public health, most notably because of their ability to cause disease in animals and humans [1, 2]. In Latin America, TBR caused by *Rickettsia rickettsii* is the most important zoonosis transmitted by ticks, with a high mortality in untreated cases [2, 3]. In Panama, TBR has been implicated in two outbreaks, separated by nearly 60 years. The first affected five people and caused two fatalities during 1950–1952 [4–6], and the second cluster of cases occurred from 2004 to 2014, when seven people were infected and six fatalities occurred [7–9]. In both outbreaks, *R. rickettsii* was the species involved. The most recent outbreak instigated new studies to determinate the distribution of TBR in rural areas and were identification of *R. rickettsii* and *Candidatus* “*Rickettsia amblyommii*”

in ticks collected from domestic mammals [10, 11].

However, in Panama there are no studies that demonstrate the presence of rickettsiae in the wilderness environment, even though the greatest diversity of ticks occurs in forests [12]. The presence of TBR in the environment around the Panama Canal Basin (PCB) was recently reported by a seroprevalence survey [13] and included the description of one human case [9], but the identities of the species of ticks vectoring these infectious agents have not been resolved.

In this paper we present new data about the presence of *Rickettsia* in questing ixodid ticks from the natural environments around the PCB.

METHODS

Study area. The PCB covers 1,474 km² (Fig. 1), encompassing one of the most biodiverse areas in the country

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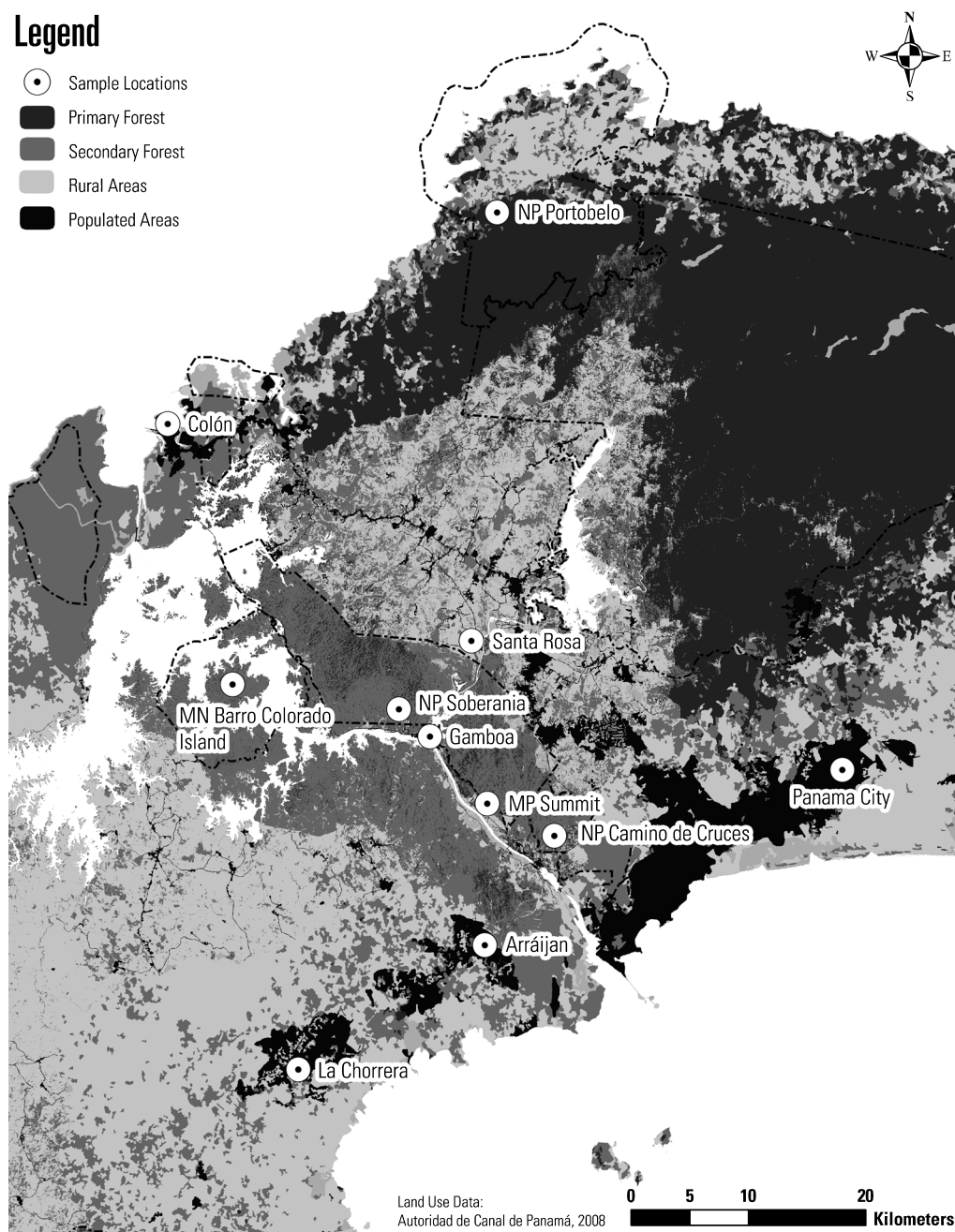


Fig. 1. Map of the study area.

[14–16]. It includes conditions that support a wide diversity of tick-host interactions [17]. Annually, this area is visited by thousands of people undertaking eco-tourism activities, and it surrounds some of the most important urban areas of the Republic of Panama, including the cities of Panama and Colon [18]. Four sites were established in native forests, characterized by the presence of mature secondary tropical rain forests (Soberania and Portobelo National Parks, Metropolitan Natural Park and Summit

Municipal Park). In addition, two riparian areas near to the towns of Clayton and Achiote were included. The sites were visited monthly from February 2009 to December 2012, during the morning hours, 0800 to 1200.

Tick sampling. A 100 m² plot was established in each area and a white cloth (45 × 45 cm) was dragged along the leaf-litter and vegetation inside the plot. This method permits the capture of ticks that climb on vegetation (e.g. *Haemaphysalis juxtakochi*, *Amblyomma tapirellum*) or that

actively seek hosts (e.g. *Amblyomma* immature); but it is inefficient in catching species showing other types of behavior (e.g. *Ixodes* spp, *Amblyomma nodosum*). Ticks that crawled onto the cloth were manually collected, deposited in 95% ethanol, and moved to the Department of Medical Entomology of the Gorgas Memorial Institute of Health Studies, for identification and counting. Taxonomic keys were used for identifying adults of *Amblyomma*, *Ixodes* and all stages of *Haemaphysalis* [12], and *Amblyomma* nymphs [19]. However, because the larvae and nymphs of some species of *Amblyomma* have not been described, some immature ticks were identified only at the genus level. Additionally, we followed the taxonomic criteria proposed by Nava *et al.* [20] for designation as *Amblyomma mixtum* within the *Amblyomma cajennense* species group.

Molecular analysis. Ticks were separated by species and site; adults were processed individually, while immature ticks were analyzed in pools of 10 larvae and 5–7 nymphs. We used Qiagen DNeasy Blood and Tissue extraction kit, following the manufacturer's instructions. Extracted DNA was stored at –20°C for later use. We used 5 µl of DNA for PCR analysis, and used the primers CS-78 and CS-323 (GCAAGTATCGGTGAGGATGTAAT and GCTTCCTTAAAATTCAATAAATCAGGAT), which amplify a 401-bp fraction of a portion of the citrate synthase gene (*gltA*), as an initial test, following the suggestion of Labruna *et al.* [21]. Amplification was confirmed by gel electrophoresis on 1% agarose, followed by and staining with ethidium bromide using a 100 bp ladder. Amplicons with expected band size were purified using Agar Ace (Promega). Direct cycle sequencing was performed on each clean PCR reaction and then sequenced in an automatic sequencer (Applied Biosystems, model ABI Prism 3130xl Genetic Analyzer, California, US). Partial sequences were subjected to BLAST analysis to determine similarities to other *Rickettsia* species.

RESULTS

We collected 7339 ticks (6981 immature ticks and 358 adults), corresponding to *Amblyomma dissimile* (seven specimens), *Amblyomma mixtum* (72), *Amblyomma naponense* (45), *Amblyomma oblongoguttatum* (84), *Amblyomma pecarium* (seven), *Amblyomma sabanerae* (two), *Amblyomma tapirellum* (36), *Haemaphysalis juxtakochi* (101), *Ixodes affinis* (four), and unidentified immature *Amblyomma* and *Ixodes* spp.

DNA was only extracted from 96 adults ticks (18 *A. mixtum*, 16 *A. naponense*, eight *A. oblongoguttatum*, two *A. pecarium*, 29 *A. tapirellum* and 23 *H. juxtakochi*) and 146 immature pools (47 larvae and 99 nymphs). Six adults

were positive for rickettsiae DNA by PCR, corresponding to five *A. mixtum* and one *H. juxtakochi*. Three and nine pools of larvae and nymphs of *Amblyomma* were positive for rickettsiae DNA, in addition to two pools of *H. juxtakochi* nymphs.

Out of these 22 PCR-positive samples, 20 were successfully sequenced for the *gltA* gene (377 bp). All 20 sequences were 99.5–100% identical to corresponding sequences of *C. "R. amblyommii"* in GenBank (DQ517290.1, CP003334.1). Two sequenced data were submitted to GenBank under the accession numbers KM654281 and KM652482. The prevalence of *C. "R. amblyommii"* in ticks is shown in Table 1 as percentage and minimum infection rate (MIR) of ticks in a pool with detectable *Rickettsia*.

No rickettsiae DNA were detected in *A. naponense*, *A. pecarium*, *A. oblongoguttatum* or *A. tapirellum*.

DISCUSSION

The questing phases comprise almost 90% of the life cycle of ticks [22–23]. In these phases, rickettsiae infection must occur before the molt or when the infection is passed from the engorged female to offspring, implicating transstadial and trans-ovarial transmission. Thus, studies focusing on questing ticks may allow a better understanding of the relationship between ticks and rickettsiae, than those devoted to ticks parasitizing hosts. To our knowledge, this is the first report of *C. "R. amblyommii"* infesting free-living *H. juxtakochi*, it provides new data regarding *A. mixtum*, which may demonstrate trans-stadial transmission into these species. It is important to note that these species are among the most common tick species in the studied area [17] and also the most likely to bite humans [24].

Amblyomma mixtum was found mainly in riparian forest, with few ticks collected in secondary or primary forest. Before the reinstatement of *A. mixtum* in Panama [20], all records of this species were mentioned as *A. cajennense*. Thus, it is possible that *A. mixtum* correspond to the former report of *A. cajennense* infected with *R. rickettsii* [6] and *C. "R. amblyommii"* from rural areas of Panama [10, 11]. In the rural setting, horses and cattle have been considered the preferred hosts for *A. mixtum* in Panama [12]; however, during the present study no domestic animals were observed, suggesting that other vertebrate are likely involved in the ecology of *C. "R. amblyommii"* at PCB. In this area, ponchos [25] and white-tailed deer seem to be the main hosts of *A. mixtum*. Hence, further research is necessary to determine the role of these mammals in the maintenance of TBR ecology in PBC.

In contrast to *A. mixtum*, *H. juxtakochi* prefer second-

Table 1. *Candidatus* “*Rickettsia amblyommii*” infection in free-living ticks from Panama. Values are given as infected ticks/individuals tested (%) for adults and positive pools/individuals tested (MIR in %) for immatures.

Species	Locality					
	Riparian Forest		Forest			PNP
	Clayton	Achiote	MNP	SMP	SNP	
Adults¹						
<i>Amblyomma mixtum</i>	1/3 (33.3)	2/6 (33.1)	1/4 (25)	0/3 (0)	—	1/2 (50)
<i>Amblyomma naponense</i>	—	—	—	0/15 (0)	—	0/1 (0)
<i>Amblyomma oblongoguttatum</i>	—	—	0/3 (0)	0/3 (0)	—	0/2 (0)
<i>Amblyomma pecarium</i>	—	—	—	0/2 (0)	—	—
<i>Amblyomma tapirellum</i>	—	—	—	0/13 (0)	0/16 (0)	—
<i>Haemaphysalis juxtakochi</i>	0/2 (0)	—	0/2 (0)	1/9 (11.1)	0/6 (0)	0/4 (0)
Immature²						
<i>Amblyomma</i> sp. (larvae)	0/1 (0)	0/1 (0)	0/6 (0)	0/9 (0)	3/15 (2)	0/1 (0)
<i>Amblyomma</i> sp. (nymph)	1/2 (8.3)	0/3 (0)	2/14 (2.8)	2/26 (1.3)	4/20 (3.4)	0/6 (0)
<i>Haemaphysalis juxtakochi</i> (larvae)	—	—	0/3 (0)	0/3 (0)	0/7	0/1 (0)
<i>Haemaphysalis juxtakochi</i> (nymph)	0/1 (0)	—	0/1 (0)	2/14 (2.4)	0/9 (0)	0/3 (0)

¹ Adults samples were analyzed for individuals. In parenthesis, the percentage of adults infected.

² Immature ticks were analyzed in pools of 10 larvae and 5–7 nymphs. In parenthesis, the MIR corresponded to the number of positive pools/number of individuals examined.

ary and primary forest [12, 26], environments with a more diverse community of potential hosts than riparian forests. In general, adults of both tick species are considered to be parasites of ungulates [12, 27]; however, again there is little information available about the immature hosts. The great diversity of vertebrates in PCB, the incomplete information about the hosts of *H. juxtakochi*, and the absence of serologic evidence of wild mammals infested with TBR make it difficult to determine the relationships among wildlife, ticks and *C. “R. amblyommii”*.

Candidatus “*R. amblyommii*” has a wide distribution in the Americas, reported in seven countries and detected in nine species of ticks [3, 28]. Hitherto, its pathogenicity has not been completely demonstrated in humans, although some authors have mentioned that it might cause mild fevers or rash at the site of the tick bite [29–32]. The effects in other mammals seem to demonstrate low infection levels. Laboratory rabbits and guinea pigs inoculated with *C. “R. amblyommii”* did not develop illness [28, 33, 34]. Other recent information has confirmed that previous infection of *C. “R. amblyommii”* is protective against infection with the more virulent *R. rickettsii* in laboratory guinea pigs [35]. *Candidatus* “*R. amblyommii*” can also induce immunological reaction in dogs and horses, without the animal presenting signs of illness [11, 36, 37]. Moreover, experimental models showed a successful trans-ovarial transmission of *C. “R. amblyommii”* at least in *A. americanum* [38], which could explain the high rates of *C. “R. amblyommii”* in other tick species [39–41].

Because antigens of many species of TBR may exhibit cross-reaction in serological tests, some authors have remarked on the possibility of *C. “R. amblyommii”* interfering in clinical diagnosis of TBR [28]. This fact is important to consider with regard to the many reports of TBR seroprevalence in Summit Municipal Park personnel [13].

Finally, even though neither *R. rickettsii* nor any other pathogenic rickettsia was detected in this study, our findings present a first approximation on the presence of rickettsiae in wooded areas of Panama. In addition, more studies that include vertebrates are required to determine the potential risk of contracting TBR in these environments.

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CONFLICT OF INTEREST

All authors declare no conflict of interests.

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