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Ticks (Acari: Ixodidae) and Associated Pathoge Collected From Domestic Animals and Vegetation in Stann Creek District, Southeastern Belize, Central America

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Abstract

Data on the prevalence and distribution of ticks and tick-borne diseases in Belize are lacking. Ticks (n = 564) collected from dogs, horses, and vegetation in two villages in Stann Creek District in southeastern Belize in 2018, were molecularly identified and screened for tick-borne nonviral human pathogens. The identity of 417 ticks was molecularly confirmed by DNA barcoding as *Rhipicephalus sanguineus* (Latreille) (66.43%), *Amblyomma ovale* Koch (15.59%), *Dermacentor nitens* Neumann (11.51%), *Amblyomma* sp. ADB0528 (3.6%), and the remainder being small records (2.87%) of *Amblyomma coelebs* Neumann, *Amblyomma imitator* Kohls, *Amblyomma tapirellum* Dunn, *Amblyomma auricularium* Conil, and *Amblyomma maculatum* Koch. Individual tick extracts were screened for the presence of *Rickettsia* spp., *Babesia* spp., *Babesia microti*, *Borrelia* spp., *Ehrlichia* spp., and *Anaplasma* spp. using available conventional polymerase chain reaction (PCR) assays. *Rickettsia parkeri* strain Atlantic Rainforest was identified in five specimens of *A. ovale*, and one other unidentified tick, all collected from dogs. Another unidentified tick—also collected from a dog—tested positive for an undefined but previously detected *Ehrlichia* sp. With the exception of *D. nitens*, all eight other tick species identified in this study were collected on dogs, suggesting that dogs could be usefully employed as sentinel animals for tick surveillance in Belize.

Key words: tick, tick-borne pathogen, *Rickettsia*, *Ehrlichia*, Belize

Although studies on ticks and tick-borne pathogens have been reported from Belize (Cline 2016, Lopes et al. 2016, Polsomboon et al. 2017), Costa Rica (Barrantes-González et al. 2016, Campos-Calderón et al. 2016, Troyo et al. 2016), Guatemala (Teglas et al. 2005), Honduras (Novakova et al. 2015), Nicaragua (Düttmann et al. 2016), Panama (Bermúdez et al. 2009, 2016; Eremeeva et al. 2009), and Mexico (Sosa-Gutierrez et al. 2016, Sánchez-Montes et al. 2019), detailed knowledge of tick ecology, distribution, and respective roles in disease transmission is very limited across Central America. Rickettsial infections are the most commonly reported tick-borne disease in Central America. Ticks infected with species of *Ehrlichia*, *Anaplasma*, and *Rickettsia* have been sampled from dogs (*Canis familiaris*) in Costa Rica (Barrantes-González et al. 2016, Campos-Calderón et al. 2016). Although *Rhipicephalus sanguineus* (Latrielle) is by far the most common tick species infesting domestic dogs in Central America, lesser numbers of *Amblyomma mixtum* Koch, *A. ovale* Koch, *Amblyomma spp*., and *Ixodes boliviensis* Neumann have also been reported (Teglas et al. 2005, Campos-Calderón et al. 2016, Düttmann et al. 2016, Troyo et al. 2016). To date, *Dermacentor nitens* Neumann and *A. mixtum* (reported as *A. cajennense* complex in Polsomboon et al. 2017) are the most common ticks collected on horses (*Equus caballus*) (Düttmann et al. 2016, Polsomboon et al. 2017).

Belize is a tropical country with rich, diverse landscapes ranging from lowlands and swamps in the north, to rainforests and mountainous regions in the south. Twenty-one species of ticks belonging to five genera—*Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Ixodes*, and *Rhipicephalus*—have been reported from environmental sampling in vegetation, and feeding on wild and domestic animals in Belize (Varma 1973, Rainwater et al. 2001, Lopes et al. 2016, Nava et al. 2017, Polsomboon et al. 2017). These include: *Amblyomma pacae* Aragão, *A. ovale*, *A. coelebs*, *A. oblongoguttatum* Koch, *A.* nr *oblongoguttatum*, *A. auricularium*, *A. dissimile* Koch, *A. longirostre* Koch, *A. mixtum* (*A. cajennense* complex), *A. tapirellum*, *A. calcaratum* (Neumann), *A. nodosum* Neuman, *A. pecarium* Dunn, *A. sabanerae* Stoll, *I. affinis*, *I.* nr *affinis*, *I. luciae* Sénevet, *D. nitens*, *R. microplus*, *R. sanguineus*, and *Haemaphysalis juxtakochi* Cooley. Most of these species also parasitize humans (Estrada-Peña and Jongejan 1999), increasing the risk of zoonosis. For example, tick-borne rickettsioses have been reported in ticks in Belize (Lopes et al. 2016 [ex. wild animals], Polsomboon et al. 2017 [ex. domestic animals]), and *Borrelia burgdorferi*—the causative agent of Lyme disease—has been found in *A. maculatum* and *D. nitens* (Cline 2016).

The aim of this study was to document the identity and distribution of tick species and ascertain associated pathogens in specimens collected from domestic animals (dogs, horses), and vegetation in the undersampled Stann Creek District of southeastern Belize. These results are critical for determining tick-borne disease risk, and developing tick management strategies that will benefit both human and animal health in southern Belize.

Materials and Methods

Study Areas and Tick Collection

Ticks were collected in Red Bank (16.618963° N, −88.558996° W) and San Roman (16.657131° N, −88.472139° W)—forest-edge villages in the Stann Creek District of, southeastern Belize (Fig. 1). With the owner's consent, attached (feeding) ticks were removed from 106 domestic dogs and three horses using forceps, and questing ticks were collected from surrounding vegetation by dragging using a standard 58 × 114 cm sailcloth sheet (BioQuip Products, Rancho Dominguez, CA). All ticks were stored in 70% ethanol prior to processing.

Nucleic Acid Extraction

DNA was individually extracted from 564 ticks using the BioSprint 96 DNA Blood Kit (QIAgen, Valencia, CA), according to the manufacturer's instructions. DNA extracts were stored at −20°C until molecular identification of tick species and associated tick-borne bacterial pathogen screening was conducted.

Molecular Identification of Tick Species and Associated Pathogens

DNA barcoding employing a 604 bp amplicon of the mtDNA cytochrome oxidase I gene was carried out to confirm species identity (see Polsomboon et al. (2017) for detailed protocol; see Table 1 for primers). Ticks were individually screened for bacterial pathogens (*Anaplasma*, *Babesia*, *Borrelia*, *Ehrlichia*, *and Rickettsia*, as well as specific primers for *Babesia microti*) using generic primers and conventional PCR (Table 1). Pathogen detection PCRs were run in 10 µl volumes, comprising 1 µl of DNA template, and 9 µl of MasterMix (1 µl BioLine 10X NH_4 buffer, 0.4 µl Magnesium Chloride [$MgCl_2$], 0.2 µl dNTP at 10mM, 0.3 µl DMSO, 0.3 µl each primer [forward and reverse] at 10 µM, 0.06 µl BioLine BIOTAQ DNA polymerase [BioLine USA Inc., Taunton, MA], and 6.44 µl nuclease free water). All amplicons were bidirectionally sequenced using the original PCR primers on an ABI 3730 sequencer.

Rickettsia-positives detected using the *OmpA* primers were subject to further confirmation using *Rickettsia* species-specific primers for *OmpB* gene. Two µl of the *OmpA Rickettsia* positive tick gDNA were added to 23 µl of the reaction mastermix, containing Phusion Flash High-Fidelity PCR Master Mix (ThermoFisher, CA), and 0.3 µM of forward (120-M59F) and reverse (OmpB1570R) primers as previously described (Jiang et al. 2013). Positive and negative controls comprising *Rickettsia africae* genomic DNA and molecular biology grade water (Gibco) were run alongside the samples. PCR products were run on 1.0% agarose gels, and products visualized with GelRed. Prior to sequencing, PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden Germany) and the sequencing reactions were cleaned using the Performa DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD). Bidirectional sequencing was performed using the original PCR primers, plus two more primers (120-607F and 120-807R) to ensure whole fragment coverage as previously described (Jiang et al. 2013).

Sequence Analysis and Voucher Specimens

Resultant raw chromatograms for both tick and pathogens were edited using Sequencher v.5.4.1 (Genes Codes Co., Ann Arbor, MI), and CodonCode aligner (CodonCode Corporation, MA). The consensus sequences were compared with publicly available sequences in GenBank, using the NCBI nucleotide BLAST search engine (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and those available on the Barcode of Life Data System (BOLD, http://www.boldsystems.org/).

Consensus DNA sequences, edited chromatograms, and associated pathogens, ecological and geolocality data for this project are publicly available on the project 'BELTC: Ticks of Belize 2019' on BOLD and sequences are available in GenBank under the accession codes ON134063–479. Tick and pathogen distribution data is included in VectorMap (http://vectormap.si.edu).

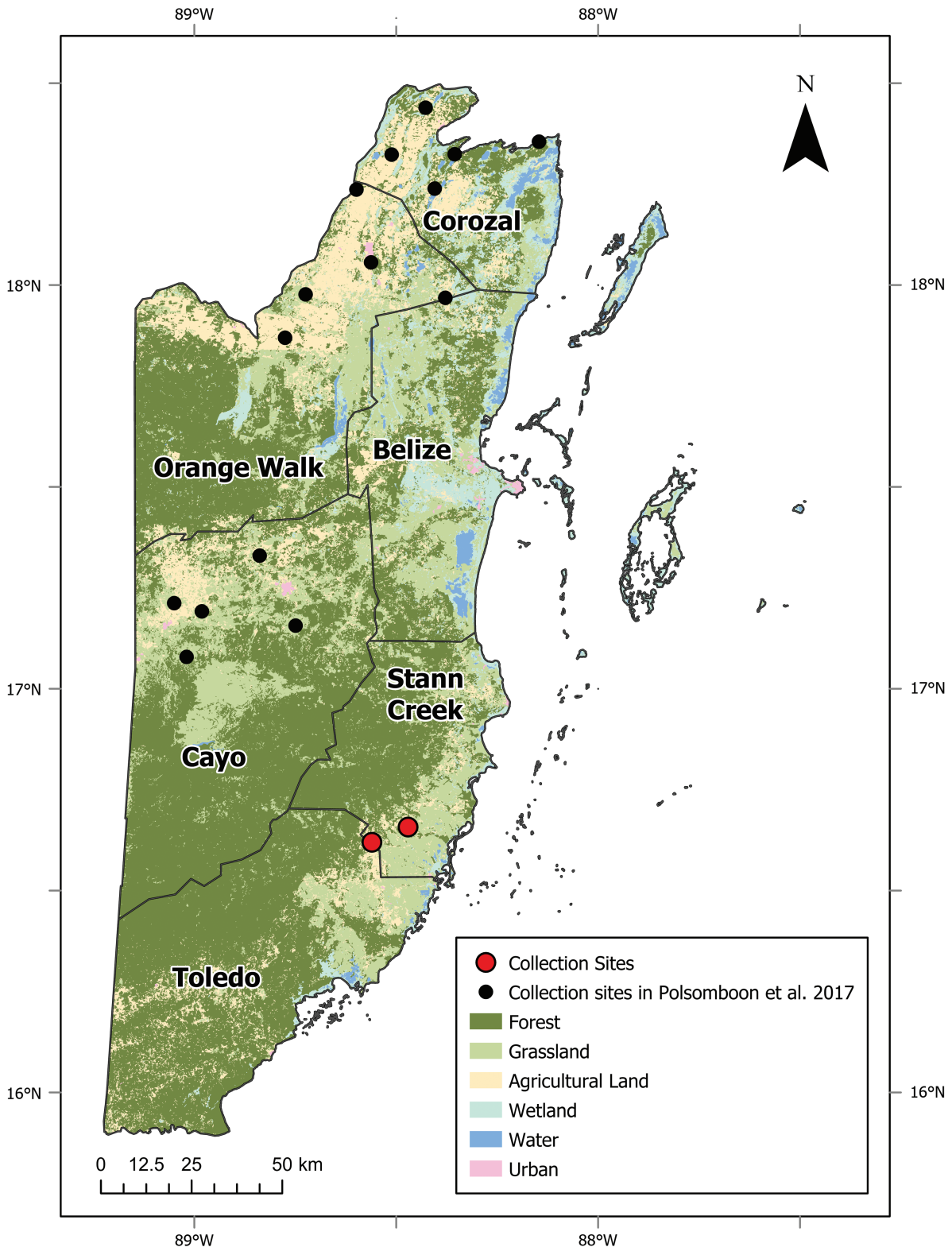

Fig. 1. Map of study collection sites in Stann Creek, Belize in comparison to those previously reported in Polsomboon et al. (2017).

Results

Tick Specimens

In total, 564 ticks were collected from two rural villages in Stann Creek District in 2018–488 from dogs, 32 from horses, and 44 from vegetation drags. Village dogs sampled in this study freely roam nearby pastures and forest areas, and interact closely with humans. DNA barcodes were successfully recovered from 417 of the 564 ticks tested (73.9%); but 564 all were screened for pathogens.

Using BLAST analysis, sequence similarity of >98% to reference sequences were considered a species match. Species matches

determined through BLAST comparisons were corroborated using the Barcode Index Number (BIN) assignments on BOLD (Ratnasingham and Hebert, 2013). In total, nine tick species were collected and identified (in order of abundance): *R. sanguineus* (*n* = 277, 66.43% of total identified; BIN# AAU2924), *Amblyomma ovale* (*n* = 65; 15.59%; BIN# AAU2925), *Dermacentor nitens* (*n* = 48; 11.51%; BIN# AAL1450), *A. imitator* (*n* = 4; BIN# ACF7338), *A. tapirellum* (*n* = 2; BIN# AAH6683), *A. auricularium* (*n* =1; BIN# ABY3174), *A. coelebs* (*n* = 4; BIN# ADB7364; *Amblyomma* sp. BZ2016 of Polsomboon 2017), and *A. maculatum* (*n* = 1; BIN# AAZ0398) (Table 2). Sequences recovered from another 15 ticks grouped together (BIN# ADB0528), most closely matching KF200150 (=*A. oblongoguttatum* specimen from Panama) at 91.2% similarity. These 15 sequences are therefore referred to herein as *Amblyomma* sp. ADB0528 (Table 2). Only *D. nitens* (*n* = 27) and *A. tapirellum* (*n* = 1) were collected off horses and both *D. nitens* (*n* = 21) and *A. ovale* (*n* = 1) were collected from vegetation, while all species but *D. nitens* were collected from dogs.

Detection and Identification of Pathogens in Ticks

Of the 564 tick specimens screened for species of *Anaplasma*, *Babesia* (and *Babesia microti*), *Borrelia*, *Ehrlichia*, and *Rickettsia*, pathogens were detected in only 14 samples (2.48%) comprising *A. ovale* (*n* = 6), *D. nitens* (*n* = 1), *R. sanguineus* (*n* = 1), and six unidentified ticks. No pathogens were detected in specimens identified as *A. auricularium* (*n* = 1), *A. coelebs* (*n* = 4), *A. imitator* (*n* = 4), *A. maculatum* (*n* = 1), *Amblyomma* sp. ADB0528 (*n* = 15), and *A. tapirellum* (*n* = 2). Of the 14 PCR-positives, seven positives collected from dogs were confirmed by sequencing and BLAST analysis. Five *A. ovale* and one unidentified tick were positive for *R. parkeri* strain Atlantic Rainforest (GenBank: OmpA = ON148411–16; OmpB = ON107292–96). One other unidentified tick collected feeding on a dog was positive for an undetermined *Ehrlichia sp.* (GenBank: ON107291), as summarized in Table 3. A further seven ticks that yielded PCR-positives for *Babesia* spp., *Babesia microti*, and *Borrelia* spp. in the initial screenings could not be confirmed by sequencing. No co-infections were detected (Table 3).

The specific infection proportions of *Rickettsia* spp. in *A. ovale* and unidentified ticks collected from dogs were 7.81% and 0.68%, respectively, and 0.68% for *Ehrlichia sp.* in unidentified ticks collected from dogs.

Discussion

Tick taxonomy is notoriously difficult, particularly in the immature stages, and molecular identification using DNA barcoding based

Table 1. Primers used for molecular identification of tick species and pathogens screening from ticks in Belize

Target gene	Primers	Sequence (5′–3′)	Size (bp)	Annealing temp	Reference
Tick *COI*	Chelicerate forward 1	TACTCTACTAATCATAAAGACATTGG	660	48°C	Barrett and Hobert 2005
	Chelicerate reverse 1	CCTCCTCCTGAAGGGTCAAAAAATGA			
Rickettsia Outer membrane	Rr 190.70p	ATGGCGAATATTTCTCCAAAA	650	51.5°C	Roux et al.1996
Protein A (*OmpA*)	Rr 190.701	GTTCCGTTAATGGCAGCATCT			
Rickettsia Outer membrane	120-M59F	CCGCAGGGTTGGTAACTGC	1,501	60°C	Roux and Raoult 2000
	ompB1570R	TCGCCGGTAATTRTAGCACT			Jiang et al. 2013
Protein B (*OmpB*)	120-607F	AATATCGGTGACGGTCAAGG			Roux and Raoult 2000
	120-807R	CCTTTTAGATTACCGCCTAA			
Babesia spp.	B-lsu-F	ACCTGTCAARTTCCTTCACTAAMTT	150	55°C	Qurollo et al. 2017
COI	B-lsu-R2	TCTTAACCCAACTCACGTACCA			
Babesia microti	Bmic-F	TTGCGATAGTAATAGATTTACTGC	230	53.1°C/52.6°C	Qurollo et al. 2017
COI	B-lsu-R2	TCTTAACCCAACTCACGTACCA			
Borrelia spp. 16S rRNA	Bor16S3F	AGCCTTTAAAGCTTCGCTTGTAG	148	57.6°C	Parola et al. 2011
	Bor16S3R	GCCTCCCGTAGGAGTCTGG			
Ehrlichia and *Anaplasma* 16S rRNA	EHR16SD	GGTACCYACAGAAGAAGTCC	345	52.8°C	Nazari et al. 2013
	EHR16SR	TAGCACTCATCGTTTACAGC			

Table 2. Molecular identification of ticks in Stann Creek District, Belize compared with publicly available *COI* sequences in GenBank and BOLD BIN

Tick species	No. of ticks	% Similarity with GenBank sequences(accession number)	BOLD BIN
A. auricularium	1	99.5% (KF200137)	ABY3174
A. coelebs	4	99.8% (MH513216, MH513218)	ADB7364
A. imitator	4	99.8% (KX360351)	ACF7338
A. maculatum	1	99.8% (KX360344, KM839245)	AAZ0398
A. ovale	65	>99.3% (KF200143, KF200079)	AAU2925
A. tapirellum	2	99.84 (KP247503)	AAH6683
Amblyomma sp. ADB0528[a]	15	91.2% (KF200150)	ADB0528
D. nitens	48	100% (MH513243, KY441486)	AAL1450
R. sanguineus	277	99.8% (KT906186, KF200112)	AAU2924

[a]Referred to herein as *Amblyomma* sp. ADB0528 which is the assigned BIN # in the BOLD database.

Table 3. Tick species and molecular detection of pathogens in ticks in Stann Creek District, Belize

Tick species	Host species	No. ticks tested	PCR positive for pathogens	Verified pathogen identification
A. auricularium	*C. familiaris*	1	0	–
A. coelebs	*C. familiaris*	4	0	–
A. imitator	*C. familiaris*	4	0	–
A. maculatum	*C. familiaris*	1	0	–
A. ovale	*C. familiaris*	64	6 (9.38%)	*R. parkeri*[a] (*n* = 5)
	Vegetation	1	0	–
A. tapirellum	*C. familiaris*	1	0	–
	E. caballus	1	0	–
Amblyomma sp. ADB0528	*C. familiaris*	15	0	–
D. nitens	*E. caballus*	27	1 (3.70%)	Unidentified
	Vegetation	21	0	–
R. sanguineus	*C. familiaris*	277	1 (0.36%)	Unidentified
Unidentified ticks	*C. familiaris*	121	6 (4.96%)	*R. parkeri*[a] (*n* = 1) *Ehrlichia* sp. (*n* = 1)
	E. caballus	4	0	–
	Vegetation	22	0	–

[a]*Rickettsia parkeri* strain Atlantic Rainforest.

on the mitochondrial *COI* gene is increasingly popular (Lv et al. 2014, Zhang and Zhang 2014, Ondrejicka et al. 2017, Polsomboon et al. 2017, Gou et al. 2018). Available *COI* reference barcodes for ticks from Belize are limited to only those 154 northern specimens (representing nine species) from our earlier study (Polsomboon et al. 2017). Herein we report an additional 417 *COI* barcode sequences, molecularly confirming the presence of nine species belonging to three genera, including *A. auricularium*, *A. coelebs*, *A. imitator*, *A. maculatum*, *A. ovale*, *A. tapirellum*, *Amblyomma* sp. ADB0528, *D. nitens*, and *R. sanguineus* in the southeastern Belizean District of Stann Creek. The current study has increased the number of known Belizean tick species with available reference DNA barcodes to 61.90% (13/21), significantly improving the future molecular identification and vector incrimination of tick species in Belize, and Central America as a whole.

Based on collection records from this and a previous study (Polsomboon et al. 2017) ticks of the species *A. ovale*, *D. nitens*, and *R. sanguineus* were commonly found parasitizing dogs and horses across Belize. In northern Belize additional species records include *A. mixtum* (previously reported as *A. cajennense* complex), *A.* nr *maculatum*, and *I.* nr *affinis*. Whereas ticks limited to southern Belize include *A. auricularium*, *A. coelebs*, *A. imitator*, *A. maculatum*, *A. tapirellum*, and *Amblyomma* sp. ADB0528 (Lopes et al. 2016 reported as *A.* cf. *oblongoguttatum*).

In our current study, *R. parkeri* strain Atlantic rainforest was detected in five *A. ovale* ticks collected from dogs. This is consistent with the findings of previous studies, where *R. parkeri* strain Atlantic rainforest was detected in *A. ovale* from wild animals in the southern Stann Creek District (Lopes et al. 2016), and *R. parkeri* was detected in *A. maculatum* from domestic dogs in Cayo District (Polsomboon et al. 2017). Additionally, *R. parkeri* strain Atlantic rainforest was reported in *A. ovale* in Argentina (Lamattina et al. 2018), Brazil (Barbieri et al. 2014, Luz et al. 2016), Colombia (Londoño et al. 2014), and Mexico (Sánchez-Montes et al. 2019). Of concern, *A. ovale* feeds readily on humans in central Panama (Bermúdez et al. 2012) and many other countries in South America (Guglielmone et al. 2006), highlighting the opportunity for zoonotic transfer of this pathogen via tick bites. A case of spotted fever rickettsiosis caused by *R. parkeri* strain Atlantic rainforest, was diagnosed in a patient bitten by an infected *A. ovale* in Brazil (da Paixão Sevá et al. 2019), and *R. parkeri* was reported in *Amblyomma* species from dogs in Latin America (Maggi and Krämer 2019), suggesting dogs may serve as a reservoir host for this pathogen. In northern and southwestern districts of Belize, *R. amblyommatis* was the most common SFG *Rickettsia* detected in ticks from domestic animals (Polsomboon et al. 2017). It has been reported from multiple tick species throughout Central America (Bermúdez and Troyo 2018), but was not detected in this study.

Multiple specimens of *R. sanguineus* and *D. nitens* appeared PCR-positive for *Ehrlichia* spp. by genus-diagnostic PCR, but only one unidentified tick collected from a dog could be confirmed as *Ehrlichia* sp. positive by direct sequencing. Both *E. canis* and *E. chaffeensis* have been detected in a variety of *Amblyomma*, *Dermacentor*, *Haemaphysalis, Ixodes*, and *Rhipicephalus* species in Latin America (Bermúdez et al. 2009, Campos-Calderón et al. 2016, Sosa-Gutierrez et al. 2016, Maggi and Krämer 2019). *R. sanguineus* has been previously found infected with *Rickettsia* species in northern Belize (Polsomboon et al. 2017) and with *E. canis*, *Anaplasma phagocytophilum*, and *R. rickettsii* in Central America and Mexico (Pat-Nah et al. 2015, Peniche-Lara et al. 2015, Campos-Calderón et al. 2016). Similarly, *D. nitens* was previously found positive for *Borrelia burgdorferi* and *Rickettsia rickettsia* in Belize (Cline 2016) and Panama (Bermúdez et al. 2009), respectively, and is considered a vector of several *Babesia* species (Schwint et al. 2008, Brites-Neto et al. 2015). However, none of these pathogens were detected in any of the ticks from the current study.

Together with our prior study (Polsomboon et al. 2017), the results from our study provide valuable additional data documenting the distribution of ticks and tick-borne bacterial pathogens in Belize, Central America. The presence of *R. parkeri* strain Atlantic rainforest and *Ehrlichia sp.* in ticks associated with free-roaming dogs living in proximity to human dwellings in rural villages may increase the risk of tick bites and tick-borne diseases in humans co-existing with these dogs. Additionally, nine of twenty-one (42%) of all reported tick species of Belize were collected off village dogs in our study, suggesting that these animals could be useful sentinels for tick surveillance in Belize. The data generated herein will augment tick management, prevention, and control programs in Belize and significantly improves tick barcode libraries for Central America, for broader scientific community reference. Additional research is still needed to better characterize tick

species, document associated pathogens, and determine tick-host associations in the region.

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