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PREVALENCE OF *BABESIA* SPP., *EHRLICHIA* SPP., AND TICK INFESTATIONS IN OKLAHOMA BLACK BEARS (*URSUS AMERICANUS*)

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ABSTRACT: American black bears (*Ursus americanus*) are commonly infested with ticks throughout their range, but there are few surveys for tick-borne disease agents in bears. To characterize tick infestations and determine the prevalence of current infection with *Babesia* spp. and past or current infection with *Ehrlichia* spp. in newly re-established populations of black bears in east central and southeastern Oklahoma, we identified adult (n=1,048) and immature (n=107) ticks recovered from bears (n=62). We evaluated serum and whole blood samples from a subset (n=49) for antibodies reactive to, and characteristic DNA fragments of *Ehrlichia* spp. as well as characteristic DNA fragments of *Babesia* spp. *Amblyomma americanum*, the most common tick identified, was found on a majority (56/62; 90%) of bears and accounted for 697/1,048 (66.5%) of all ticks recovered. Other ticks included *Dermacentor variabilis* (338/1,048; 32.3%) from 36 bears, *Amblyomma maculatum* (9/1,048; 0.9%) from three bears, and *Ixodes scapularis* (4/1,048; 0.4%) from three bears. Antibodies reactive to *Ehrlichia* spp. were detected in every bear tested (49/49; 100%); maximum inverse titers to *Ehrlichia chaffeensis* ranged from 64–4,096 (geometric mean titer 1,525). However, PCR failed to identify active infection with *E. chaffeensis*, *Ehrlichia ewingii*, or an *Ehrlichia ruminantium*-like agent. Infection with *Babesia* spp. was detected by PCR in 3/49 (6%) bears. Together these data confirm that tick infestations and infection with tick-borne disease agents are common in bears in the southern US. The significance of these infestations and infections to the health of bears, if any, and the identity of the *Ehrlichia* spp. responsible for the antibody reactivity seen, warrant further evaluation.

Key words: *Babesia* spp., black bear, *Ehrlichia* spp., tick, *Ursus americanus*.

INTRODUCTION

American black bears (*Ursus americanus*) are native to Oklahoma but were eliminated during the early 1900s through unregulated hunting and habitat destruction (Clark and Smith 1994). Successful reintroduction of black bears in the Ouachita and Ozark Mountains in Arkansas in the 1950s and 1960s resulted in recolonization into Oklahoma in the 1980s (Bales et al. 2005). Bear numbers have substantially increased in eastern Oklahoma in the last 2 decades (Pfander 2016). Although recent work has documented that black bears in Missouri host large numbers of ticks and likely play a role in both maintenance and dispersal of several ixodid species (Al-Warid et al. 2017), little information is available about tick infestations in the Oklahoma populations and there is a dearth of data on tick-borne infections in bears in the region.

Ticks are commonly found on black bears throughout most of their range in North America (Yabsley et al. 2009; Al-Warid et al. 2017), suggesting infection with tick-borne agents may be equally prevalent. Serologic or molecular evidence of tick-borne infections, including *Anaplasma phagocytophilum*, *Babesia* spp., *Borrelia burgdorferi*, and *Rickettsia* spp., has been reported from black bears in the northeastern or far western US (Bronson et al. 2014; Chern et al. 2016), but surveys of tick-borne agents in bears in the central US are lacking, and no study has yet reported

evidence of past or current infection with *Ehrlichia* spp. in bears.

As part of ongoing efforts to determine the status and distribution of black bears in eastern Oklahoma by the Oklahoma Department of Wildlife and Conservation (ODWC), we collected ticks and blood samples from sedated, trapped bears to characterize the most-common ticks that infested the Oklahoma black bear population as well as the prevalence of past or current infections with *Ehrlichia* spp. and *Babesia* spp.

MATERIALS AND METHODS

Bears were trapped between May–August 2014 by teams consisting of Oklahoma State University students and ODWC personnel using snare or barrel and culvert traps in the Ozark Plateau and Ouachita Mountains of eastern Oklahoma. Trapped bears were chemically immobilized with a mixture of tiletamine/zolazepam (Telazol, A.H. Robbins Co., Richmond, Virginia, USA) and xylazine (xylazine, RXV, Greeley, Colorado, USA) at a dose of 4.8–7.0 mg/kg prior to examination. Teeth were collected for cementum annuli age estimations. To minimize immobilization time, only a representative sample of ticks was collected from each bear; variable collection times precluded detailed assessment of infestation intensity. Additional tick collections were carried out in February–March 2015 on female bears and their cubs in their dens. Ticks were removed with forceps and placed in 70% ethanol. All ticks were enumerated and identified to stage and species in the laboratory by comparison to standard keys (Keirans and Litwak 1989). Whole blood was collected from bears directly into vacuum tubes containing either no additive or ethylenediaminetetraacetic acid anticoagulant and placed on ice for transport to the laboratory. Serum was harvested by centrifugation at 1,500 × G and aliquots of serum and whole blood stored at –20 C until tested. All animal procedures were approved by the Oklahoma State University's Institutional Animal Care and Use Committee (Protocol AG-13-6) prior to initiation of these studies.

Serum samples from 49 bears for which samples were available were evaluated for antibodies to *Ehrlichia* spp., *B. burgdorferi*, and *Anaplasma* spp. and antigen of *Dirofilaria* spp. using a commercial enzyme linked immunosorbent assay (ELISA; IDEXX SNAP® 4Dx™Plus, IDEXX Laboratories Inc., Westbrook, Maine, USA) according to the manufacturer's instructions. Archived serum samples (n=10), from North American brown bears (*Ursus arctos*), from a region of Canada where *Ehrlichia* spp. are not known to cycle in nature, were included as negative controls. Immunofluorescent assays (IFA) to detect antibodies reactive to *Ehrlichia chaffeensis* also were performed using commercially available slides (Fuller Laboratories, Fullerton, California, USA) as previously described (Schultz et al. 2002).

Nested PCR was performed on blood samples from 49 bears. Briefly, nucleic acid was extracted (Illustra™ blood genomicPrep Mini Spin Kit, GE Healthcare, Pittsburgh, Pennsylvania, USA) from each whole blood sample or from ticks (n=46) and used as a template in previously described nested PCR assays designed to amplify characteristic 16S rDNA fragments of *E. chaffeensis* and *Ehrlichia ewingii* or characteristic 18S rDNA fragments of *Babesia* spp. (Gubbels et al. 1999; Little et al. 2010). An additional single-step PCR was performed to detect characteristic *glt*A fragments of Panola Mountain *Ehrlichia* as previously described (Qurollo et al. 2013). Direct sequencing of *Babesia* spp. amplicons was performed (SimpleSeq™, Eurofins MWG Operon Inc., Huntsville, Alabama, USA). Due to the presence of multiple 18S rRNA gene sequences in samples, amplicons were prepared for cloning (Wizard PCR Preps, Promega Corporation, Madison, Wisconsin, USA), cloned using the Invitrogen TOPO® TA Cloning® Kit (Life Technologies, Carlsbad, California, USA), and plasmids extracted using the Promega PureYield™ plasmid MiniPrep Kit (Promega Corporation). Plasmids with correctly sized inserts were sequenced with an ABI 3730 capillary sequencer (Applied Biosystems, Foster City, California, USA) at the Oklahoma State University Molecular Core Facility (Stillwater, Oklahoma, USA). Molecular phylogenetic analysis was performed on the sequences of the cloned samples using the maximum likelihood method based on the Tamura 3-parameter model in MEGA version 6.0 (Tamura et al. 2013).

The geometric mean number of ticks was calculated based on the count +1 transformation of the actual numbers for each species, with 1 subsequently subtracted from the results. Confidence intervals (CI) for geometric means were based on natural log-transformed values. We calculated 95% modified Wald CI for each proportion and used unpaired t-tests with significance assigned at 0.05 to evaluate the association between sex and age of bear and tick infestations and the number of ticks recovered from each bear in the two populations (QuickCalcs, GraphPad Software, Inc., San Diego, California, USA).

TABLE 1. Species, number, geometric mean number per bear, and minimum prevalence of adult ticks collected from 62 American black bears (*Ursus americanus*) examined in 2014 in Oklahoma, USA.

Species	Total	Number of adult ticks collected				Minimum prevalence		
		Percent	GM[a]	95% CI[b]	Range	Median	Percent	95% CI
Amblyomma americanum	697	66.5	6.4	4.6–8.6	0–88	8	90	80.1–95.8
Dermacentor variabilis	338	32.3	2.3	1.4–3.5	0–34	1	58	45.7–69.5
Amblyomma maculatum	9	0.9	0.06	0–0.1	0–6	0	4.8	1.1–13.8
Ixodes scapularis	4	0.4	0.04	0–0.1	0–2	0	4.8	1.1–13.8
Total	1,048						100	93.0–100

[a] GM = Geometric mean number of ticks per bear (calculated instead of intensity of ticks because timing constraints of chemical immobilization and handling did not allow for complete examination).
[b] 95% confidence interval.

RESULTS

A total of 62 active black bears were examined from May through August, 2014, including 23 males and 37 females; sex was not recorded for two bears. The bears ranged from 1–18 yr of age (mean: 5.4 yr, 95% CI: 4.4–6.5); females were an average of 6.4 yr old (range: 1–18) and males were an average of 4.1 yr old (range: 1–11). Age was not determined for 11 bears. Ticks were present on every bear (Table 1). *Amblyomma americanum*, the most-common tick found, was identified from 56/62 bears (90%) and accounted for 697/1,048 (66.5%) adult ticks (145 females, 552 males) followed by *Dermacentor variabilis* (338/1,048; 32.3%; 133 females, 205 males), which was found infesting 36/62 bears (58%). *Amblyomma americanum* was the most-common tick recovered in May and June and *D. variabilis* was the most-common tick found in July and August. *Amblyomma maculatum* and *Ixodes scapularis* were each recovered from three bears. Immature *A. americanum* (13 larvae and 94 nymphs) were identified from 18 bears, most of which (16/18, 89%) were also infested with adult *A. americanum*. Tick infestations did not differ significantly by sex (P=0.713) or age class (juvenile versus adult, P=0.355) of bear. Significantly more *A. americanum* were recovered from bears from the Ozark Plateau than from bears in the Ouachita Mountains (t=4.23, df=60, P=0.001); numbers of other tick species recovered were not significantly different between the two populations. A total of 35 denning bears (13 females and 22 cubs) were examined in February and March 2015. No ectoparasites were found on the cubs and two male *A. americanum* and one male *D. variabilis* were found on three female bears (one tick on each).

Whole blood and serum samples were available from 49 of the active bears. All (49/49, 100%) serum samples were positive for antibodies to *Ehrlichia* spp. by commercial ELISA; no antibodies to *B. burgdorferi* or *Anaplasma* spp. were detected, and no black bears had evidence of antigen of a *Dirofilaria* sp. Antibody to *Ehrlichia* spp. was not detected in brown bear (0/10, 0%) sera. Brown bear sera were also negative for antibodies to *B. burgdorferi* and *Anaplasma* spp., although six of 10 brown bear sera had antigen of a *Dirofilaria* sp. All black bear samples were also positive for antibodies reactive to *E. chaffeensis* on IFA, and titers ranged from 1:64 to 1:4,096, with a geometric mean inverse titer of 1,525 (95% CI: 1,111–2,093). A majority (37/49, 76%) of black bear sera had inverse titers ≥1,024, and maximum titers were seen in bears with varied numbers of ticks recovered, including those from which relatively few adult *A. americanum* were collected.

No characteristic fragments were amplified by nested PCR of whole blood for *E. chaffeensis*, *E. ewingii*, or Panola Mountain *Ehrlichia* spp. Sequence-confirmed 16S rRNA gene fragments ≥99.7% similar to *E.*

chaffeensis (AF147752) were identified in 2/46 (4%) adult *A. americanum* ticks removed from bears.

Nested PCR revealed *Babesia* spp. infection in 3/49 bears (6%), each with a distinct sequence. One bear harbored a *Babesia* sp. sequence that was ≥99.8% identical to a *Babesia* sp. (KR017880) reported from a maned wolf (*Chrysocyon brachyurus*), another had a *Babesia* sp. sequence ≥99.1% similar to *Babesia microti* from a raccoon (*Procyon lotor*) from Massachusetts, USA (AY144701), and the third had a *Babesia* sp. sequence ≥99.6% similar to a *Babesia* sp. from a raccoon from Illinois, USA (DQ028958).

DISCUSSION

Ticks are common on black bears throughout North America. Indeed, surveys have documented infestations in nearly every population of black bears examined (Al-Warid et al. 2017). In the present study, *A. americanum* was found on >90% of the bears examined, and bears were also infested with *D. variabilis*, *A. maculatum*, and *I. scapularis*. These findings are not surprising as *A. americanum* is the most-common tick reported from most of the southern US (Barrett et al. 2014; Duell et al. 2013). The wide home range of black bears may allow them to be involved in the expansion of tick populations within a given region, particularly in woodland habitats (Lyda et al. 2007; Al-Warid et al. 2017).

The predominance of *A. americanum* and *D. variabilis* on bears in the present study was likely accentuated by active collection from May through August when adults of these two species of ticks are most active. Based on activity peaks for the respective species in the region, earlier collections could have resulted in recovery of more *A. maculatum*, and later collections in the fall may have revealed more *I. scapularis* (Koch 1982). Moreover, the average number of ticks recovered from each bear in the present study almost certainly represents an underestimation. Examinations for ticks were somewhat limited by the time constraints of the immobilization and the need to prioritize other required monitoring activities; more complete examinations, such as those performed at necropsy, would have likely resulted in collection of a greater number of ticks, particularly immature stages. Due to this limitation, the prevalence of tick infestation for the minor tick species reported in the present study should only be viewed as minimum prevalence; nonetheless, ticks were present on every bear examined (Table 1).

The universal presence of ticks on the active bears also leads to questions as to whether other tick species may be feeding on bears in the winter months in Oklahoma. Previous work has shown that *I. scapularis* was the most-common tick on bears in the northeastern US in the fall and spring; although more work is needed to document host competency over time, black bears may contribute to supporting *I. scapularis* populations where both the bears and ticks are present (Zolnik et al. 2015). The interactions of ticks and denning bears, however, are not well described in the literature. In the only study to examine denning bears in winter, one of eight hibernating black bears studied in the upper Midwest was infested with *Dermacentor albipictus* ticks (Rogers 1975). In the present study, the lack of ticks on denning bears in the winter months when *I. scapularis* would be active may have indicated any ticks acquired by bears in the fall had already completed feeding and detached. Alternatively, this finding could have been due to the den location, the lack of mobility of bears in the later fall months in which they acquired their ticks, the lack of nidicolous behavior among most hard tick species, or the challenge presented by thick winter fur both for ticks to feed and for quick searching strategies deep inside the den. Further work on this topic, particularly collection of ticks from bears in October and November when both bears and *I. scapularis* remain active, would be of value.

Reports of tick-borne infections in the expanding populations of black bears from the southern US are largely limited to testing of attached, fed ticks, an approach that may not accurately reflect infection in the bears (Yabs-

ley et al. 2009). In areas where *I. scapularis* is the dominant tick species, *B. burgdorferi*, *A. phagocytophilum*, and *B. microti* infections are reported from black bears (Zolnik et al. 2015; Chern et al. 2016), but research is largely lacking in areas where additional tick species are found. Recent work has documented antibodies to *B. burgdorferi* and *R. rickettsii*, but not *Ehrlichia* spp., in black bears in northern Maryland (Bronson et al. 2014), and an earlier study showed antibodies to *A. phagocytophilum* in black bears in Pennsylvania (Schultz et al. 2002). However, the present paper is the first report of antibodies to true *Ehrlichia* spp. in American black bears. This finding is not surprising in light of the universal tick infestation we documented and the common prevalence of *Ehrlichia* spp. infection in other mammals infested with ticks in this region (Carmichael et al. 2014; Starkey et al. 2014). We were also not surprised that all bears were serologically negative for *B. burgdorferi* and *A. phagocytophilum*, as neither agent is known to cycle in the region (Little et al. 2014).

Although PCR was not successful at identifying the *Ehrlichia* sp. responsible for inducing the antibody titers, IFA using *E. chaffeensis* as antigen confirmed the initial findings by commercial ELISA. Moreover, none of the brown bear sera from an area where *A. americanum* ticks are not known to be present harbored antibodies reactive to *Ehrlichia* spp. on either commercial ELISA or IFA, suggesting that both assays were performing as expected. Indeed, although serologic cross-reactivity cannot be entirely excluded, seroprevalence to *E. chaffeensis* in white-tailed deer (*Odocoileus virginianus*) in the southern US is usually 100% in areas with adequate *A. americanum* populations, and antibodies are detected much more frequently than molecular detection of rickettsemia by PCR (Yabsley et al. 2005).

Infections with *Babesia* spp. have been previously reported from Japanese black bears (*Ursus thibetanus japonicas*; Ikawa et al. 2011) and from American black bears in New Jersey, USA (Shaw et al. 2015; Zolnik et al. 2015). Two of these sequences were >99% identical to those reported from raccoons in other areas of the US (Birkenheuer et al. 2006) while a third differed by only one base substitution to a *Babesia* sp. reported from a captive maned wolf (*Chrysocyon brachyurus*; KR017880). It is unknown if a *Babesia* sp. infection in black bears poses any health risks to the bears. Additionally, the role, if any, of American black bears as a wildlife reservoir for potentially pathogenic *Babesia* spp. of domestic animals and humans remains unclear (Shaw et al. 2015).

Interestingly, antigen of a *Dirofilaria* sp., presumably *Dirofilaria ursi*, was detected in six of 10 brown bears used as negative control sera for cross-reactive antibodies to *Ehrlichia* spp. but in none of the black bears in the present study. *Dirofilaria ursi* is considered uncommon in black bears in the southeastern US, although this parasite is commonly reported from *Ursus* spp. in Canada and Alaska (Dies 1979), where it is transmitted by blackflies (Simuliidae) and has occasionally been associated with zoonotic disease (Beaver et al. 1987). Cross-reactions of *D. immitis* antigen detection kits have been reported when these assays are used to test samples from animals infected with some other nematodes including *Angiostrongylus vasorum*, *Spirocerca lupi*, and *Acanthcheilonema odendhali* (Schnyder and Deplazes 2012; Aroch et al. 2015; Krucik et al. 2016).

Taken together, these data suggest that black bears in the southcentral states are likely exposed to or infected with *Ehrlichia* spp. in nature to a greater extent than previously thought and can be infected with a variety of *Babesia* spp., suggesting further research into tick-borne infections in the expanding populations of American black bears in the southern US is warranted. Additional sampling may allow closer examination of the effect of bear longevity or tick infestation levels on the prevalence of infection with tick-borne pathogens in bears.

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