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# Detection and Prevalence of *Babesia* spp. in American Black Bears (*Ursus americanus*) from Eastern and Western North Carolina, USA

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ABSTRACT: Blood samples collected from American black bears (*Ursus americanus*) in eastern and western North Carolina, US, were analyzed for piroplasms. Piroplasmids were detected in 17% (23/132) of the animals surveyed. We detected a *Babesia* spp. previously identified in North American raccoons (*Procyon lotor*) and a maned wolf (*Chrysocyon brachyurus*); prevalence was 22% (14/64) and 13% (9/68) in the mountain and coastal black bear populations, respectively. The presence of the same *Babesia* species in black bears, raccoons, and a maned wolf suggests piroplasms may not be host specific.

*Key words:* American black bear, *Babesia*, North Carolina, PCR, piroplasms, *Theileria*, *Ursus americanus*.

Piroplasmid transmission occurs mostly through competent vectors or direct contact (i.e., animal fights; Ikawa et al. 2011). Specifically, *Babesia* transmission is commonly associated with ticks of the order *Ixodida*, and the most commonly recognized vectors are *Dermacentor* spp., *Rhipicephalus* spp., *Amblyomma* spp., and *Ixodes* spp. (Hodžić et al. 2017). American black bears (*Ursus americanus*) provide blood meals for ticks throughout the majority of their range (Yabsley et al. 2009; Chern et al. 2016; Skinner et al. 2017). Black bear populations and human-bear interactions in North Carolina, US, have increased over the past 35 yr for many reasons. Therefore, the objective of our study was to determine the molecular prevalence of piroplasms in two populations of free-ranging black bears in eastern and western North Carolina.

A total of 132 blood samples from black bears were analyzed. Postmortem blood was collected from 68 bears (47 males and 21 females) in eastern North Carolina from Beaufort, Hyde, Tyrrell, and Washington counties during the November 2015 regulated hunting season, and from 64 bears (29 males and 35 females) from western North Carolina that were live-trapped, sampled, and released between April 2014 and September 2015 in Asheville, Buncombe County, North Carolina (Fig. 1). Live bear studies were approved by the Institutional Animal Care and Use Committee at North Carolina State University and followed American Society of Mammalogists Guidelines (Gannon et al. 2007).

All bears were in good body condition with no abnormalities on physical exam. Ages were determined by tooth annuli. The median body weight of eastern North Carolina bears was 134 kg (interquartile range: 90–210 kg), and ages ranged between 1.75 and 15.75 yr old. Blood samples from eastern North Carolina bears were collected from major vessels from 30 min up to 8 hr after death during carcass processing. Ticks (attached and unattached) were collected from carcasses. Blood (4 mL) from western North Carolina bears was collected from the femoral artery into tubes treated with ethylenediaminetetraacetic acid. Ectoparasites, including unattached and attached ticks, were collected from multiple anatomical locations (axillae, pinnae, and inguinal areas). Blood and ticks in alcohol from all bears were stored at –20 C until processed.

Our PCR targeted a 600-base-pair region of the *18S rRNA* gene used to detect piroplasms (*Babesia* spp. and *Theileria* spp.) in DNA extracted from blood samples as previously reported (Varanat et al. 2011). The concentration of DNA was measured using the

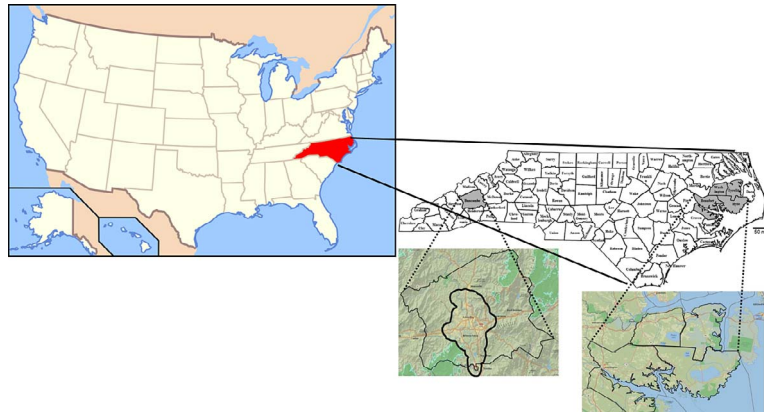

FIGURE 1. Hunting areas (wide black lines) in Asheville, Buncombe County (western North Carolina, USA) and Beaufort, Hyde, Tyrell, and Washington counties (eastern North Carolina) where black bears (*Ursus americanus*) were sampled in 2014–15.

absorbance ratio between 260 and 280 nm (NanoDrop, ThermoFisher Scientific, Waltham, Massachusetts, USA) after DNA extraction from 200 µL of blood from each bear with a QIAmp DNA Mini Kit (Qiagen Inc., Valencia, California, USA).

Piroplasmid DNA was amplified using a piroplasma 18S-144s: 5′ ACCGTGCTAATTG TAGGGCTAATACA 3′ (forward primer) and a piroplasma 18S-722as: 5′ GAATGCCCC CAACCGTTCCTATTAAC 3′ (reverse primer). The DNA was amplified in 25 µL final volume containing 12.5 µL Tak-Ex® Premix (ThermoFisher Scientific), 7.3 µL molecular grade water, 0.2 µL of each primer (100 pmol/µL; IDT DNA Technology, Coralville, Iowa, USA), and 5 µL of DNA extracted from bear blood. Molecular-grade water (5 µL) was used for negative controls. Positive controls were prepared with 5 µL of DNA from blood of a dog infected with *Babesia canis*. An Eppendorf Mastercycler EPgradient® (Eppendorf, Hauppauge, New York, USA) was used to complete conventional PCR using a single hot-start cycle at 95 C for 3 min followed by 55 cycles of denaturing at 94 C for 15 s, annealing at 68° C for 15 s, extension at 72 C for 18 s, and amplification at 72 C for 2 min. A 2% agarose gel electrophoresis and visualization using ethidium bromide under ultraviolet light was used to analyze all PCR products. DNA sequences were aligned and edited with AlignX (Vector NTI suite 11.5.1, Invitrogen, Carlsbad, California, USA). Samples were sequenced by Eton Bio, Inc. (Research Triangle Park, North Carolina, USA). Evolutionary analyses were constructed in MEGA7 (Kumar et al. 2016). The Maximum Likelihood method, based on the Tamura-Nei model (1,000 bootstrap replications), was used for molecular phylogenetic analysis.

No DNA amplification was detected in any negative extraction control or PCR negative control sample. Overall, 17% (23/132) of blood samples were positive for piroplasm DNA amplification: 22% (14/64) and 13% (9/68) from western and eastern North Carolina bears, respectively. Four groups of piroplasms were identified by DNA sequence analysis comparing the 18SrRNA amplified region: Group A, from a single bear from western North Carolina showing a homology of 100% (588 base pairs) with a *Babesia* sp. (GenBank accession no. DQ028958) detected in a raccoon from Illinois (Birkenheuer et al. 2006); Group B, detected in 11 bears (four from western North Carolina and seven from eastern North Carolina), showing 99.8% (587/588 base pairs) homology with a *Babesia* sp. from a raccoon (GenBank no. DQ028958) and 99.7% (586/588 base pairs) with a *Babesia* sp. (GenBank no. KR017880) from a maned wolf (*Chrysocyon brachyurus*); Group C, identified in 10 bears (nine from western North Carolina and one from eastern North Carolina), showing

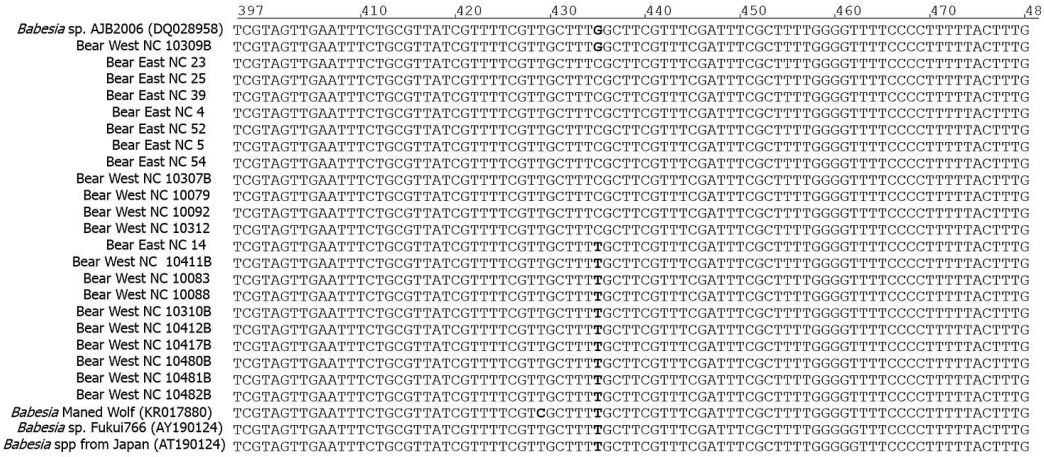


FIGURE 2. Differences in a 588-base-pair region of the piroplasma 18S rRNA region among *Babesia* species detected in black bears (*Ursus americanus*).

a 99.8% (587/588 base pair) homology with GenBank DQ028958 and GenBank KR017880 sequences; and Group D, detected in a single bear from eastern North Carolina, showing 100% (588 base pairs) homology with *Theileria cervi* isolated from elk (*Cervus elaphus*) in Wisconsin and Indiana (no. AY735134) and from a white-tailed deer (*Odocoileus virginianus*) from Oklahoma (no. AY735122).

We used real-time PCR amplification of a 150 bp region of mitochondrial genome (partial c oxidase subunit cox gene region) from *Babesia* (Qurollo et al. 2017), to assess if sequence differences in 18SrRNA detected in groups A, B, and C (Fig. 2) could have been due to different *Babesia* species. We detected no sequence differences between groups, which suggests that all three belonged to the same species (AJB-2006) detected in a North American raccoon (no. DQ028958).

Piroplasm infection was identified at an overall molecular prevalence of 17% (23/132) in 132 black bears from North Carolina. Black bears from eastern (13%, 9/68) and western (22%, 14/64) North Carolina had similar prevalence, and each was greater than those reported in Oklahoma (Skinner et al. 2017), and New Jersey (Shaw et al. 2015; Chern et al. 2016), previously.

Using 16SrRNA mitochondrial DNA sequencing techniques (Maggi et al. 2010), we identified ticks collected from black bears from western North Carolina as *Ixodes scapularis* and *Ixodes cookie*. Ticks from eastern North Carolina bears were identified using a tick identification key and included *Amblyomma americanum*, *Ixodes*, and *Dermacentor* spp.

Most *Babesia* spp. detected in black bears from eastern and western North Carolina were >99% homologous with a *Babesia* spp. previously identified in a raccoon (Birkenheuer et al. 2006) and in a captive maned wolf (Phair et al. 2012). These organisms appear to be the same species based on DNA analysis (Fig. 3). However, it is unclear if the small base-pair differences in the 18SrRNA region (described as groups A, B, and C) reflect mutations occurring because of geographic differences (separated loci) in either competent vector, host populations, or host dynamics, or if they represent different strains of the same species.

The black bears we studied showed no clinical signs of disease. *Babesia* infection is often asymptomatic until an animal is immunosuppressed or coinfected with other vector-borne pathogens (Alvarado-Rybak et al. 2016). Further studies are needed to assess the clinical impact of piroplasms on wildlife. Our results support other studies (Ikawa et al. 2011; Shaw et al. 2015; Skinner et al. 2017) suggesting black bears can be a reservoir for these and other vector-borne pathogens. The increased bear population in North Carolina and the associated increase in contact be-

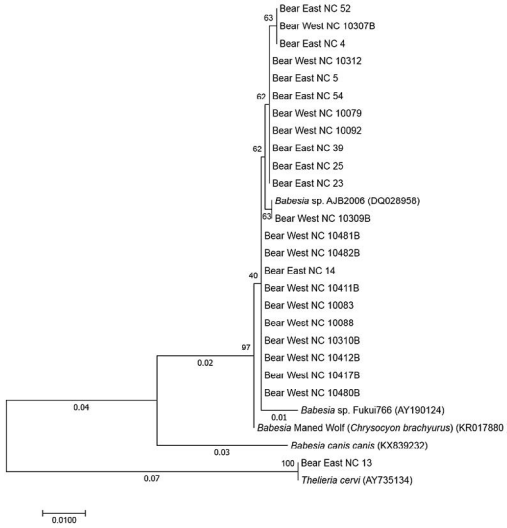


FIGURE 3. Molecular phylogenetic analysis with the Maximum Likelihood method of *Babesia* 18S rRNA sequences detected in black bears (*Ursus americanus*) from mountain and coastal North Carolina, USA in 2014–15. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (1,000 bootstrap replications). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 28 nucleotide sequences. There was a total of 621 positions in the final dataset.

tween bears, humans, vectors, and domestic animals suggest a potential for babesiosis and other vector-borne zoonosis exposure.

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