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DIVERSE *BARTONELLA* SPP. DETECTED IN WHITE-TAILED DEER (*ODOCOILEUS VIRGINIANUS*) AND ASSOCIATED KEDS (*LIPOPTENA MAZAMAE*) IN THE SOUTHEASTERN USA

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ABSTRACT: There are many known species of *Bartonella*, Gram-negative bacteria that can cause febrile illness and fatality in humans and animals. These pathogens are often transmitted through hematophagous arthropod vectors such as fleas and lice. Despite increasing awareness about *Bartonella* spp. and their zoonotic potential, as well as existing literature on *Bartonella* spp. in cervids, little is known about the diversity of *Bartonella* spp. in white-tailed deer (*Odocoileus virginianus*) and their associated keds in the southeastern US. We examined the prevalence and diversity of *Bartonella* spp. in an enclosed herd of white-tailed deer and their ectoparasites, deer keds (*Lipoptena mazamae*), in Alabama. The overall prevalence of *Bartonella* infection in this population of deer was 16% (10/63) and 24% (23/96) in keds associated with deer that we sampled. Three species of *Bartonella* were identified in both deer and their keds: *Bartonella bovis*, *Bartonella schoenbuchensis*, and *Bartonella* sp. 1. Additionally, *Bartonella melophagi* was detected in white-tailed deer but not in the sampled keds. The detection of four *Bartonella* species in one population of white-tailed deer, three of which have known zoonotic potential, highlights the importance of *Bartonella* diversity within host species.

Key words: Ectoparasite, *Lipoptena*, *Odocoileus*, pathogen, vector ecology, zoonotic.

INTRODUCTION

Bartonellosis is a vector-borne disease caused by Gram-negative bacteria of the genus *Bartonella*. These bacteria infect erythrocytes, endothelial cells, and macrophages, which can lead to persistent blood-borne infections. Bacteria of the genus *Bartonella* are variable in pathogenicity and prevalence. The taxonomy of *Bartonella* species has expanded significantly since the early 1990s, as has the number of species known to infect humans (Angelakis et al. 2010; Okaro et al. 2017). Many arthropod vectors are known to transmit *Bartonella* efficiently and cause zoonotic infections. These include sandflies (*Lutzomyia verrucarum*; Battisti et al. 2015), human body louse (*Pediculus humanus*; Kim et al. 2017), cat flea (*Ctenocephalides felis*; Chomel et al. 2006), a rodent flea (*Ctenophthalmus nobilis*; Parola et al. 2003), and the sheep tick (*Ixodes ricinus*; Reis et al. 2011). *Bartonella* has many additional suspected vectors, including other biting flies (Chung et al. 2004), fleas (Parola et al. 2003), ticks (Angelakis et al. 2010), and deer keds of the genus *Lipoptena* (Regier et al. 2018). In a previous study, 94% of collected keds and other Hippoboscidae harbored *Bartonella* DNA (Halos et al. 2004).

The deer ked (*Lipoptena mazamae*) is a fly that seeks a bloodmeal as a winged adult and then again on their hosts where they lose their wings and find mates. These vectors show adenotrophic viviparity and have only one offspring at a time. This makes keds vulnerable to transovarial infection, which is a hypothesized reason why keds are such good vectors even though they only have one offspring at a time (Härkönen and Kaitala 2013; Korhonen et al. 2015). Vertical transmission of pathogens such as *Bartonella* has been documented in the abdomen from mothers to larval keds (De Bruin et al. 2015). The wingless nature of many adult keds reduces their opportunities for feeding

on a variety of hosts and can limit their geographic mobility. The deer ked is found throughout the southeastern US and Central America (Heine et al. 2017; Skvarla and Machtinger 2019) and is associated with white-tailed deer and occasionally humans.

To examine *Bartonella* diversity in deer and associated keds, we selected a population of semiwild white-tailed deer (*Odocoileus virginianus*) located in the Piedmont region of east-central Alabama (Neuman et al. 2016; Newbolt et al. 2017) for this study, due to the high prevalence of deer-ked infestation in the population (Zikeli 2018). This semiwild population is ideal for this study because deer were present at densities approximately five times greater than that found naturally in the region and provided a unique opportunity to study *Bartonella*-host-ked dynamics.

The purpose of this study was to identify species of *Bartonella* in *O. virginanus* and their associated deer keds in the southeastern US. Deer and their keds are an important study system for understanding vector transmission dynamics because of the high infestation rates of keds on deer, the ability for keds to vector *Bartonella* spp., and the zoonotic potential of these pathogens.

MATERIALS AND METHODS

Sample collection

The Auburn University Deer Lab is a 174 ha facility (32°49′37″N, 85°38′44″W) and consists of 5.5 km of 3 m, high-tensile perimeter fencing. The animals in the facility had free-range of the entire complex, and behavioral interactions among animals were natural. Sex ratios during the time of the study were 1:1. At the time of construction, approximately 35 wild deer were enclosed in the facility, and during the study period from October to March 2016–17 and 2017–18 the population consisted of approximately 120 animals that were either the original deer or their descendants. Population numbers were maintained within the facility through natural mortality and the removal of 10–15, 6-mo-old fawns per year. A high proportion of the males in the herd were ≥3.5 yr old. Deer had access to natural forage as well as to supplemental feed (16–18% extruded protein feed; Record Rack®, Nutrena Feeds, Minneapolis, Minnesota, USA) that was available ad libitum at three gravity feeders. Additionally, four timed feeders each provided deer with approximately 2 kg/d of corn from October to March. Corn was dispensed for capture purposes and not to meet nutritional needs. Vegetation within the facility consisted of approximately 40% open fields maintained for hay production, 13% bottomland hardwoods (*Quercus* spp.), 26% mature mixed pine (*Pinus taeda*) and hardwood (*Quercus* spp. and *Carya* spp.), 11% early successional regenerating thickets (*Rubus* spp., *Liquidambar styraciflua*, *Juniperus virginiana*, and *Ligustrun sinense*), and 10%, 10–15-yr-old *P. taeda* plantation. Properties outside of the facility consisted primarily of forestland of various ages, areas of pasture maintained for hay production, approximately 80 ha of pasture with cattle, and other residential and rural land uses.

Sixty-three adult male white-tailed deer were chemically immobilized and captured from October to March in 2016–17 and 2017–18. Deer were immobilized using a combination of tiletamine-zolazepam (Telazol®, Zoetis, Parsippany, New Jersey, USA; 100 mg/mL given at a rate of 4.5 mg/kg) and xylazine (Lloyd Laboratories, Shenandoah, Iowa, USA; 100 mg/mL given at a rate of 2.2 mg/kg). Anesthetized deer were reversed with tolazoline (Tolazine®, Lloyd Laboratories; 100 mg/mL given at a rate of 6.6 mg/kg; Miller et al. 2004). Chemical immobilization was delivered using cartridge-fired dart guns (Pneu-Dart, Williamsport, Pennsylvania, USA) equipped with night vision scopes and transmitter darts at feeders (Saalfeld and Ditchkoff 2007). Once immobilized, approximately 10 mL of whole blood was collected from each deer via jugular venipuncture for later molecular analysis. A full-body count of keds was conducted using a single flea comb and fine-toothed steel forceps (Fantahun and Mohamed 2012), with the goal of collecting at least one adult ked from each captured deer. Collected keds were identified to species, sexed, and stored in 90% ethanol. All methods were approved by the Auburn University Institutional Animal Care and Use Committee (PRN 2013-2372 and 2016-2964) and followed the American Society of Mammalogists' guidelines (Sikes and Animal Care and Use Committee of the American Society of Mammalogists 2016).

Molecular techniques

A 5-mL aliquot of whole blood from each individual deer was stored in vials with ethylenediaminetetraacetic acid as anticoagulant at –20 C until DNA was extracted. The Qiagen DNeasy Blood and Tissue Extraction Kit (Qiagen Inc., Germantown, Maryland, USA) was used according to the manufacturer's instructions. Pan-*Bartonella* primers were designed using the National Center for Biotechnology and Informa-

tion's Primer-Blast tool (2012) based on the 16S rRNA gene for genus-specific detection with a 481 base pair amplicon size (forward-5′-TTAGCCGTCGGGTGGTTTAC-3′; reverse-5′-GCGATTCCGACTTCATGCAC-3′). We used 1 μL of each forward and reverse primer of 10 μM concentration in the PCR reactions. Negative controls were used for DNA extraction to evaluate potential contamination.

Using a conventional PCR protocol, 3 μL of extracted DNA was combined with a Qiagen HotStarTaq Plus Master Mix Kit (Qiagen) and nuclease-free water according to the manufacturer's instructions. Thirty-microliter aliquots of the master mix and DNA sample were placed in flat-capped stripped tubes inside a Bio-Rad T100 Thermal Cycler (Hercules, California, USA) for 35 cycles of the following program: 95 C for 5 min, 94 C for 30 s, 54 C for 30 s, 72 C for 1 min, with a final cycle of 72 C for 10 min. Ultra-pure water served as the no-template control for PCR. Gel electrophoresis was performed on a 2% agarose gel and run at a consistent 100 V for 35–40 min before being imaged. A c200 ultraviolet light (Azure Biosystems, Dublin, California, USA) was used for gel imaging. All samples with positive PCR amplicons were subsequently sequenced using Sanger sequencing (Eurofins Scientific, Val, Flueri, Luxembourg) and resulted in *Bartonella* spp. matches suggesting high specificity of this assay. All sequences were deposited in GenBank, and the accession numbers are MK951985–MK951994.

Each ked was bisected vertically, with one half used for DNA extraction (using the same method as used for the whole blood). We extracted DNA from 96 keds, a minimum of one from each sampled deer according to the manufacturer's instructions for the tissue protocol of the Qiagen DNeasy Blood and Tissue kit (Qiagen). Ectoparasites were first homogenized in a solution of 180 μL buffer ATL and 20 μL Proteinase K with the addition of eight autoclaved 6-mm zinc oxide ball bearings (BC Precision, Chattanooga, Tennessee, USA) using a bead mill homogenizer (Omni International, Kennesaw, Georgia, USA) at 4,000 revolutions per minute for 45 sec. Samples were then incubated in a thermomixer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 56 C until foam from homogenization had subsided. The remaining steps followed the manufacturer's protocol for DNA extraction. Ked extracts were processed using the same *Bartonella* primers as the blood protocol described above. Negative controls were used throughout the DNA extraction and PCR process to evaluate contamination. Positive controls were also used to validate the PCR results of positive banding. A laboratory-cultured sample of *Bartonella henselae* was used as a genus-wide positive control.

Statistical methods

Analysis was conducted using a Poisson distribution to evaluate for zero inflation in the statistical program R, version 3.0.3 (glmmTMB; R Development Core Team 2018). All models included a random term to control for the repeating sample of male deer over the two study years and to account for variation that occurs in infection over the seasonal period.

Phylogenetic analysis

Retrieved *Bartonella* sequences and reference sequences from GenBank were aligned using the Vector NTI Express software (Life Technologies, Carlsbad, California, USA). Based on these alignments, phylogenetic trees were constructed by the neighbor-joining method using the Kimura 2-parameter model. We further examined bootstrap values in which only values equal to or greater than 70% are considered valid using MEGA 6 (Tamura et al. 2013), to compare our detected *Bartonella* spp. with known sequences (Supplementary Material Fig. 1).

RESULTS

A total of 63 adult male white-tailed deer were included in this study. All individuals captured were infested, and the only ked species that we found was *L. mazamae* (Fig. 1). Whole blood was collected from each deer, and a total of 96 keds (a minimum of one per deer) were processed. Ten of the 63 blood samples tested positive for *Bartonella* spp. (16%); *Bartonella melophagi* (GenBank no. MK995240) was the most prevalent with four deer testing positive, three deer tested positive for a previously undescribed *Bartonella* sp. 1 (GenBank nos. MK951985–MK951994), two of the 10 deer tested positive for *Bartonella bovis* (GenBank no. MK951984), and one deer tested positive for *Bartonella schoenbuchensis* (GenBank nos. MK951995–MK951998; Fig. 2). Deer infected with *Bartonella* had 37% more keds ($\bar{x}$=119.6; SE=14.23; P=0.006) per individual than noninfected deer ($\bar{x}$=87.3; SE=4.34).

Keds were found around the face and neck (to the shoulder), ears (inside and outside), along the spine (5–10 cm in width on one side), and the anogenital region (the anus to base of tail, surrounding scrotum and to the femoral region), from the shoulder to the rear flank, and

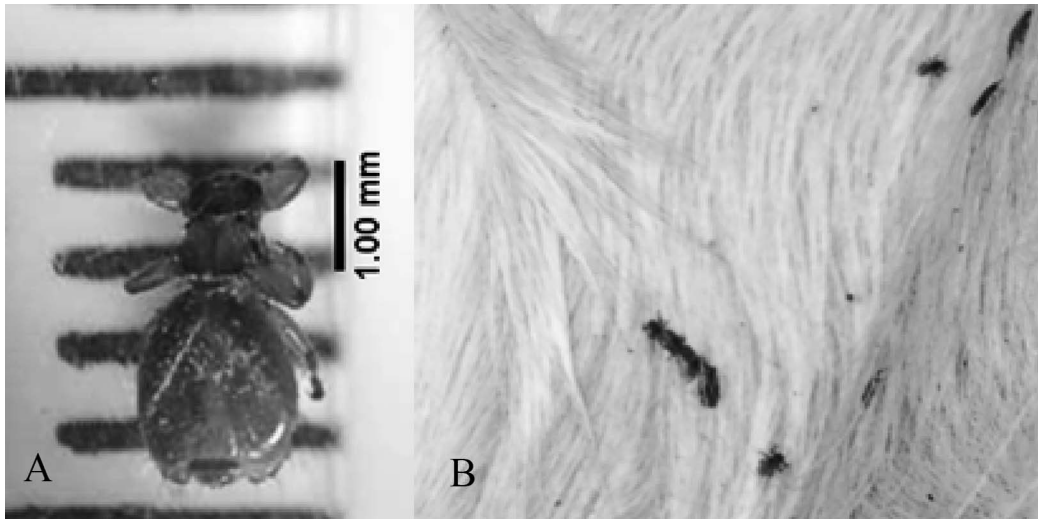

FIGURE 1. Deer keds (*Lipoptena mazamae*) were found to infest all individuals in our study population of semiwild white-tailed deer (*Odocoileus virginanus*) in an enclosure in Alabama, USA, and representative adult specimens were collected from each deer. Adult female keds were removed from white-tailed deer and sampled for *Bartonella* spp. diversity (A), although adults of both sexes were found parasitizing individuals and were often found mating on deer (B).

from the sternum to the femoral region. Among the 96 deer keds collected and analyzed, 23 were positive for *Bartonella* spp. Eight of these were from deer that were confirmed as *Bartonella* positive. The remaining 15 positive keds were from deer that did not have detectable *Bartonella* spp. DNA present in the whole blood samples. Of the positive keds, 10 were identified as *B. schoenbuchensis* (44%) at identity ≥99% (GenBank no. CP19789), eight were identified as *B. bovis* (35%) with an identity ≥99% (NR-025121), and five were identified as *Bartonella* spp. (22%) with an identity ≥99% (GenBank no. CP019781). Two of the five keds that tested positive for *Bartonella* sp. were determined to have the previously undescribed *Bartonella* sp. 1, which was also found in the deer that they parasitized (Fig. 2).

DISCUSSION

We documented the diversity of *Bartonella* species in white-tailed deer and their associated keds in the southeastern US. We anticipated that the most common *Bartonella* infection would be that of *B. schoenbuchensis* because it has previously been isolated or detected from other ruminants, such as roe deer (*Capreolus capreolus*; Dehio et al. 2004), and it has been suspected to be transmitted by a wide variety of vectors, including ticks and deer keds (Angelakis et al. 2010; De Bruin et al. 2015). However, we noted diverse *Bartonella* species in both deer and keds, including one previously undescribed species. Due to sample depletion, we were unable to attempt isolation and use additional gene targets, such as *rpoB* and *gltA*, which have been implicated as the best markers for demarcation of *Bartonella* species (La Scola et al. 2003). Therefore, the samples we refer to as *Bartonella* sp. 1 may be more conservatively considered *Bartonella* lineage 1. Future work on this population may provide a description of this lineage in greater detail.

The deer ked is a known vector of several *Bartonella* spp. (Szewczyk et al. 2017; Regier et al. 2018), especially *B. schoenbuchensis*. Detection of *B. bovis*, *B. schoenbuchensis*, and *Bartonella* sp. 1 in deer keds suggested infection but does not implicate the deer ked as a competent vector of these species. Vector competency has not yet been con-

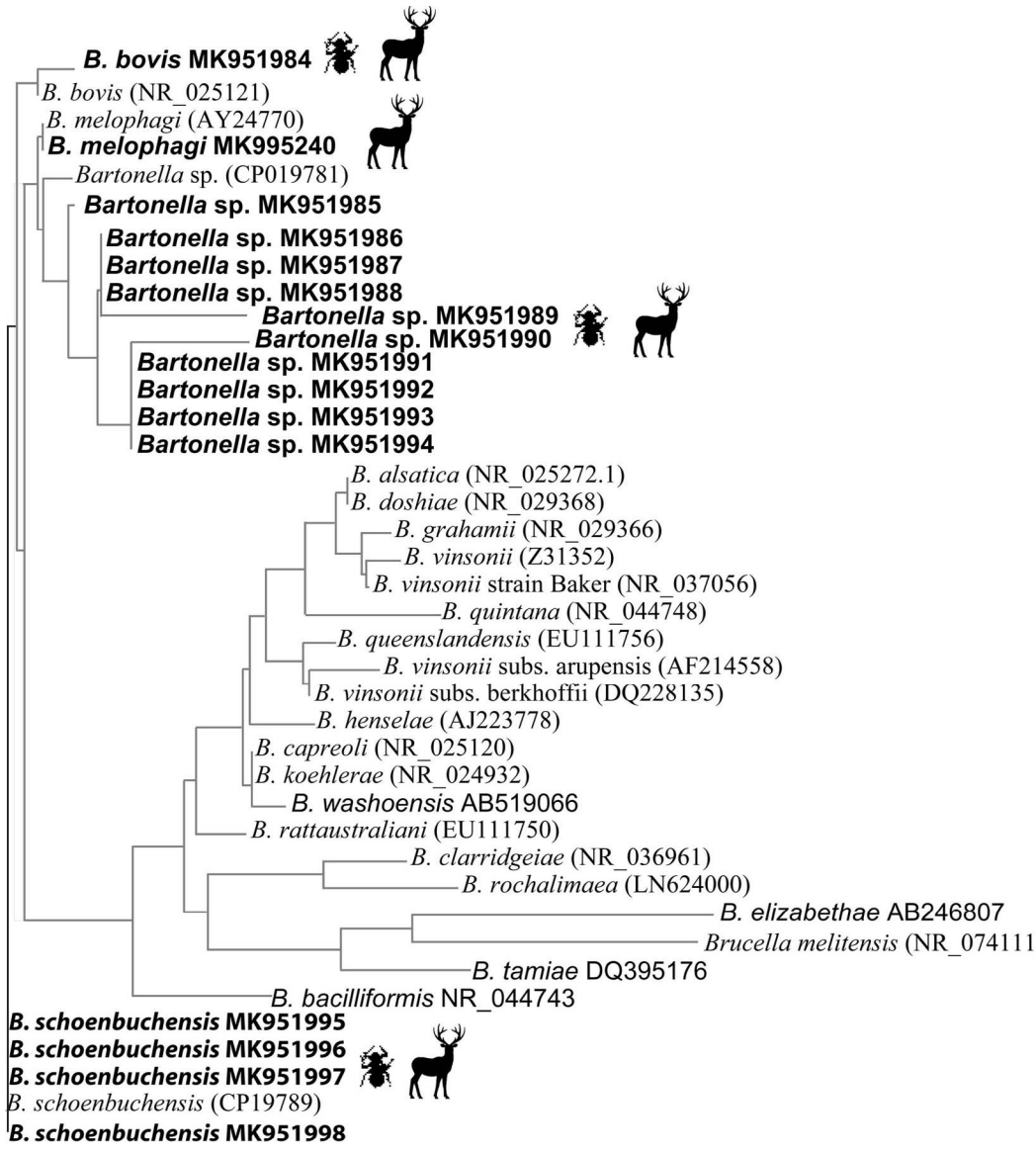

FIGURE 2. Phylogenetic tree of *Bartonella* amplicons based on sequences of the 16S *Bartonella* gene. Phylogenetic trees were constructed using the neighbor-joining method. The 33 different sequences of 16S rRNA obtained were classified into *Bartonella bovis*, *Bartonella melophagi*, *Bartonella schoenbuchensis*, and *Bartonella* sp. 1. GenBank accession numbers and bolded text represent samples in our study of *Bartonella* spp. detected in deer keds (*Lipoptena mazamae*) recovered from semiwild white-tailed deer (*Odocoileus virginianus*) in an enclosure in Alabama, USA. Text that is not bold represents phylogenetic *Bartonella* outgroups, *Brucella melitensis*, and their GenBank accession numbers. Deer and ked icons in the figure demonstrate whether each of the four species detected was found in sampled deer, keds, or both.

firmed in any hippoboscid fly or ked. Vertical transmission of *B. schoenbuchensis* occurs in the abdomen of mothers to larval keds (De Bruin et al. 2015). Little is known about the transmission dynamics of deer keds; however, it is known that they preferentially feed on deer (Välimäki et al. 2011).

Bartonella infection in deer was correlated with infestation of keds. *Bartonella*-positive keds were collected from *Bartonella*-negative

deer and vice versa, making it impossible to conclude the likelihood of transmission from this study, but it is possible that deer keds may play a role in the transmission of *Bartonella* among this deer population. We observed a positive trend of infection in relation to severity of infestation by deer keds, which may also play a role in the transmission of *Bartonella* through this system. Vertical transmission of *Bartonella* has been identified in deer keds, and this may be another mechanism through which this deer ked may perpetuate *Bartonella* through the system. It is also possible that keds could be parasitizing available host animals outside of the facility and transporting *Bartonella* into, out of, or throughout the enclosure, either through contact and direct movement of keds, vertical transmission to larvae, or through winged adults that could feed on either side of the fenced enclosure.

Our study site was a semiwild enclosure for white-tailed deer, surrounded by agricultural fields, forested habitats, and human settlements. Since *B. melophagi* and *B. bovis* are most often detected in livestock, it is possible that winged adult keds fed on animals adjacent to the enclosure before feeding on white-tailed deer within the enclosure. The enclosure's perimeter fence allowed for the passage of smaller mammals both over and through it. Camera traps located in the enclosure have captured domestic house cats (*Felis catus*), raccoons (*Procyon lotor*), bobcats (*Felis rufus*), coyotes (*Canis latrans*), nine-banded armadillos (*Dasypus novemcinctus*), eastern gray squirrels (*Sciurus carolinensis*), Virginia opossums (*Didelphis virginiana*), and other medium-sized mammals during the trapping periods of this study. These animals that can move beyond the fence and easily encounter other animals and humans that live in the area. The deer ked has wings when it emerges from its puparium but loses them once it finds a host. White-tailed deer overlap with humans and other species in Alabama, and these interactions may present opportunities for interspecific and intraspecific ked movement and opportunities for the zoonotic *Bartonella* spp. detected here to be transmitted. The high prevalence of previously undescribed *Bartonella* sp. 1 in both deer and keds in this population suggests the potential significance of this bacterium in the region. Further investigation into this *Bartonella* species may provide insight into its pathogenicity and zoonotic potential.

SUPPLEMENTARY MATERIAL

Supplementary material for this article is online at http://dx.doi.org/10.7589/2019-08-196.

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