

Research Article

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Prevalence and diversity of piroplasms and ticks in young raccoons and an association of *Babesia sensu stricto* infections with splenomegaly

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Abstract

Piroplasms are intraerythrocytic parasites that are often transmitted by ixodid ticks, but vertical transmission is an alternative route for some species. In the USA, raccoons (*Procyon lotor*) are hosts for two known species, a *Babesia microti*-like sp. and *Babesia lotori* (in *Babesia sensu stricto* group). To better understand the natural history of *Babesia* in raccoons, we tested young raccoons from Minnesota and Colorado for *Babesia* spp., examined them for ticks, and assessing for splenomegaly as a sign of clinical disease. Raccoons from both states were infected with *B. microti*-like sp. and *Babesia sensu stricto* spp. Infections of *B. microti*-like were common, even in 1-week-old raccoons, suggesting vertical transmission. *Babesia sensu stricto* infections were more common in older raccoons. Raccoons infected with *Babesia sensu stricto* had significantly higher spleen:body weight ratios compared with uninfected or *B. microti*-like sp.-infected raccoons. Ticks were only found on raccoons from Minnesota. The most common and abundant tick was *Ixodes texanus* but *Ixodes scapularis* and *Dermacentor variabilis* were also found on raccoons. We report piroplasm infections and infestations with several tick species in very young raccoons. Young raccoons infected with *Babesia sensu stricto* spp. had higher spleen:body weight ratios, suggesting a disease risk.

Introduction

The piroplasms are an important cause of disease in humans, domestic animals and some wildlife, although most piroplasms in wildlife demonstrate low pathogenicity for their natural host (Hunfeld *et al.* 2008; Yabsley and Shock, 2012). Most piroplasms with known life cycles use ixodid ticks as vectors (Hunfeld *et al.* 2008) although vertical transmission has been noted as a possible alternative transmission route for some piroplasms (e.g., *Babesia microti* in laboratory mice and humans, *Babesia gibsoni* and *Babesia canis canis* in dogs and *Babesia bovis* in cows) (Yeruham *et al.* 2003; Fukumoto *et al.* 2005; Joseph *et al.* 2012; Mierzejewska *et al.* 2014; Bednarska *et al.* 2015; Adaszek *et al.* 2016; Costa *et al.* 2016). In addition, fighting and intermixing of individuals' blood has been associated with direct transmission of *B. gibsoni* between fighting dogs (Yeagley *et al.* 2009).

Babesia infections in raccoons have been reported sporadically throughout the Eastern and Midwestern USA (Schaffer *et al.* 1978; Anderson *et al.* 1981; Telford and Forrester, 1991; Birkenheuer *et al.* 2006, 2007; Clark *et al.* 2012). However, there are currently little data on the pathogenicity of piroplasm infections in raccoons. A survey of raccoons from Japan with splenomegaly, a pathologic consequence of *Babesia* infections in other species, found that 8% (2/24) were positive for *Babesia* spp.; however, only raccoons with splenomegaly were tested (Kawabuchi *et al.* 2005; Adaszek *et al.* 2016). Reports of *Babesia* infections in raccoons are generally based on older studies that utilized blood smears for piroplasms detection (Schaffer *et al.* 1978; Anderson *et al.* 1981; Telford and Forrester, 1991).

Two species of morphologically similar, but molecularly distinct, piroplasms have been reported from Florida, Massachusetts, North Carolina and Illinois (Goethert and Telford, 2003; Birkenheuer *et al.* 2006; Birkenheuer *et al.* 2007; Clark *et al.* 2012). One is a species related to *B. microti* (hereafter called *B. microti*-like species) and the other is a *Babesia sensu stricto* species now referred to as *B. lotori* (also called *Babesia* sp. AJB-2006) (Anderson *et al.* 1981; Birkenheuer *et al.* 2007). The phylogenetic relationships of the piroplasms are under debate, but the *B. microti* clade is considered by many researchers to be a novel genus and likely has many unique biological characteristics (Lack *et al.* 2012; Schreeg *et al.* 2016). Without molecular characterization, it is unknown which or both of these species are present in infected raccoons. Furthermore, the prevalence and distribution of these two raccoon piroplasms is poorly known. Outside of the USA, *via* molecular assays, a low prevalence of *B. microti*-like parasites and at least one *Babesia sensu stricto* species have been reported from raccoons introduced to Japan (Kawabuchi *et al.* 2005; Jinnai *et al.* 2009).

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Currently, the transmission route is unknown for both *Babesia* spp. of raccoons but it is presumed to be *via* ixodid ticks as most piroplasms are transmitted by ixodid ticks (Uilenberg, 2006; Hunfeld *et al.* 2008). Raccoons are commonly infested with several tick species including *Ixodes texanus*, *Ixodes cookei*, *Ixodes scapularis*, *Ixodes affinis*, *Dermacentor variabilis*, *Amblyomma americanum* and *A. maculatum*, but the geographic distribution and seasonality of many of these tick species varies (Dennis *et al.* 1994; Ouellette *et al.* 1997; Yabsley *et al.* 2008). In North Carolina, both piroplasms are found in raccoons in high prevalences (Birkenheuer *et al.* 2006), so the vector, if there is one, is presumed to be a common tick species found on raccoons. Unfortunately, all of the tick species noted above are found in North Carolina so testing of raccoons from various parts of North America would be needed to better identify possible tick vectors (Ouellette *et al.* 1997; Birkenheuer *et al.* 2006). However, there is the possibility of alternative transmission routes.

Numerous questions remain regarding the natural history of piroplasm infections in raccoons, including the prevalence and diversity of different piroplasm species infecting raccoons, modes of transmission and pathogenicity. Thus, our objectives were to determine the prevalence of *Babesia* in young raccoons under the age of 6 weeks old and, if they occurred, what species of *Babesia* were present. We hypothesized that young raccoons would be infected with at least the two species of piroplasms previously reported in raccoons as the prevalence of both are very high in adult raccoons. In addition, we calculated a spleen:body weight ratio to determine if raccoons infected with *Babesia* spp. had larger spleens because splenomegaly has been associated with piroplasm infections. Finally, we examined the raccoons to determine if an infestation of ticks occurred while still in the nest, as raccoons younger than 6 weeks old typically do not venture from the nest (Schneider *et al.* 1971; Gehrt and Fritzell, 1998). The current vector(s) of any piroplasms of raccoons are unknown but if piroplasm infections were noted in young raccoons, *I. texanus*, a tick species that is transmitted among raccoons in the nest (Anderson *et al.* 1981), would be expected to be present and would possibly be associated with transmission. However, it is also possible that other tick species may be transmitted to young raccoons within the nest environment. We sampled young raccoons in Minnesota and Colorado where we have previously detected *Babesia* infections in adult raccoons (Garrett and Yabsley, unpublished data).

Methods

Sample collection

From April to November of 2016, samples were collected from fetal, neonatal or juvenile raccoons admitted to two rehabilitation facilities: the Wildlife Rehabilitation Center of Minnesota (Roseville, MN) and the Greenwood Wildlife Rehabilitation Center in Colorado (Longmont, CO). The raccoons sampled were presented to the centres deceased, died while in care, or were euthanized due to poor prognosis. Raccoons were frozen immediately after death to ensure no decomposition occurred and shipped to the Southeastern Cooperative Wildlife Disease Study (Athens, GA) where they were processed. Raccoons were examined for ectoparasites, which if found, were preserved in 70% ethanol until identification. Ticks were identified morphologically using published keys (Keirans and Litwak, 1989; Durden and Keirans, 1996; Guglielmono *et al.* 2014) or by using molecular methods as described below. Spleens were removed after examination, weighed and re-frozen at -20°C until testing.

Data collected from each raccoon included weight, body length, sex and estimated age based on tooth eruption

(Montgomery, 1964). For a limited number of raccoons, age was approximated based on weight because of missing or damaged teeth. The age of remaining raccoons was also estimated based on weight and both methods provided similar results (data not shown). Although no animals were euthanized for the purposes of this study, the collection of biological samples for pathogen testing was reviewed and approved by UGA's Institutional Animal Care and Use Committee (A2014 10-018).

Molecular testing

Genomic DNA was extracted from ~ 10 mg of spleen using a commercial kit per the manufacturer's instructions (DNEasy Blood and Tissue kit, Qiagen, Hilden, Germany). Two different PCR (polymerase chain reaction) assays targeting the V4 region of the 18S rRNA gene of *Babesia* were used as described (Birkenheuer *et al.* 2003, 2007). One set of primers, BMlikeF (5'-CTGCCTTATCATTAAATTCGCTTCCGAACG) and 793-772R (5'-ATGCCCCCAACCGTTCCCTATTA), targets *Babesia* parasites in the *B. microti*-like clade. Molecular analyses were conducted on a BioRad DNA Engine Peltier Thermal Cycler (Bio-Rad Laboratories Incorporated, Foster City, CA). Cycling parameters were 94°C for 5 min followed by 49 cycles of 94°C for 45 s, 56°C for 45 s and 72°C for 45 s, with a final extension of 72°C for 5 min. The other set of primers, 455-479F (5'-GTCTTGTAATTGG-AATGATGGTGAC) and 793-772R, were used to detect *Babesia sensu stricto* species. Cycling parameters were 94°C for 3 min followed by 44 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, with a final extension of 72°C for 5 min.

Precautions were taken to prevent and detect contamination including the performance of DNA extraction, PCR reaction setup, and product analysis in distinct, designated areas. Negative water controls were included in each set of DNA extractions. For each batch of PCR reactions, the extraction negative control, a new water negative control and a positive control (DNA sample from a pooled blood sample with the sequenced-confirmed presence of *B. lotori* and *B. microti*-like sp.) were included. Amplicons were observed in a GelRed stained 1.5% agarose gel. Gels were run for an extended period of time to ensure the reliable distinction between amplicon sizes.

Because the screening PCR assays amplify a small amplicon not ideal for species identification, especially within the *Babesia sensu stricto* group, 11 samples positive with the *Babesia sensu stricto* group 18S screening primer set were also tested using a PCR targeting the cytochrome c oxidase subunit 1 (*cox1*) region and products were sequenced to identify species present. Primers Babcox1F (5'-GGAAGTGGWACWGG-WTGGAC) and Babcox1R (5'-TTCGGTATTGCATGCCTTG) were used and cycling parameters were 95°C for 5 min followed by 45 cycles of 95°C for 20 s, 50°C for 30 s, 68°C for 1 min and 30 s, and a final extension of 72°C for 5 min (Schreeg *et al.* 2016). For eight samples that were coinfecting and a *Babesia sensu stricto* sequence was not obtained using the 18S screening or *cox1* gene protocols, we conducted an additional PCR using *Babesia sensu stricto*-specific primers that target two regions of the large subunit rRNA (LSU) gene fragment (*lsu5* and *lsu4*) (Quorollo *et al.* 2017).

To confirm the presence of *B. microti*-like sp. in a subset of samples the BMlikeF/793-772R amplicon was sequenced (nine samples) or a larger region of the 18S rRNA gene was amplified with primers 522F and 1661R and sequenced (four samples) (Birkenheuer *et al.* 2007).

Amplicons were purified from an agarose gel using a gel-purification kit (Qiagen) and bi-directionally sequenced at the University of Georgia Genomics Facility (Athens, GA). Sequences were cleaned using the Genomics program (Biomatters Limited,

Auckland New, Zealand) and consensus sequences compared with other *Babesia* sequences in GenBank.

Some *Ixodes* ticks were damaged during removal and could not be identified to species using morphologic characteristics. Tick DNA was extracted and amplified as described (Gleim *et al.* 2014). Primers 16S-1 (5'-CCGGTCTGAACTCAGATCAAGT) and 16S+2 (5'-TTGGGCAAGAAGACCCTATGAA) targeting the 16S rRNA gene were used and cycling parameters were 94 °C for 2 min followed by 40 cycles of 94 °C for 30 s, 45 °C for 30 s, 72 °C for 1 min and a final extension of 72 °C for 5 min. Amplicons were sequenced as described above.

Statistical analyses

A Fisher's exact test was used to compare the prevalence of the *B. microti*-like sp. and *Babesia sensu stricto*. In order to determine the relationships between parasite prevalence and the different variables, a generalized linear model (GLM) was utilized and the data log transformed. The variables measured included sex, age (by week), spleen:body weight ratio and whether or not ticks were present. A separate GLM was performed for the *B. microti*-like sp. and *Babesia sensu stricto* datasets.

Results

A total of 106 young raccoons from Minnesota ($n=83$) and Colorado ($n=23$) were included in the study and 66% (70/106) were infected with *Babesia*. The prevalence of *B. microti*-like sp. [66/106 (62%)] was significantly higher than that of *Babesia sensu stricto* [11/106 (10%) ($P=0.0001$)]. A total of eight (7.5%) raccoons had coinfections. For the *B. microti*-like sp., infections were detected in individuals as young as 1 week of age and prevalence was high in all age groups (Table 1). For the *Babesia sensu stricto* group, infections were first noted at 2 weeks of age but prevalence was low, while prevalence in raccoons that were 6 weeks or older was 40% (Table 1). Table 2 shows the data for the 59 raccoons from Minnesota that were admitted in identifiable litters. In general, the prevalence of *Babesia* within litters was high but for most litters, not all individuals were infected (Table 2).

Eight of the 11 *Babesia sensu stricto* samples amplified with the cox1 PCR protocol but only five provided good quality sequences

(all from Minnesota). One sequence was most similar (99.8%) to a *Babesia* sp. reported from a captive maned wolf (*Chrysocyon brachyurus*) (KR017881) but was also 98.3% similar to *Babesia lotori* (accessioned as *Babesia* sp. AJB-2006, KR017882) of raccoons. Two other sequences, from the same litter of raccoons, were identical to each other and, although they were most similar to *B. lotori*, they only shared 84.2% similarity (Table 2). The remaining two sequences were most similar (90.4%) to *B. vulpes* (a *B. microti*-like sp. of fox and dogs, accessed in GenBank as *Babesia* sp. MES-2012, KC207827); however, these sequences were identical to unpublished sequences of the *B. microti*-like sp. of raccoons (Garrett and Yabsley, unpublished data). Both of these raccoons were coinfecting with a *Babesia sensu stricto* based on the screening 18S PCR; one (16-5935) of which had a partial 18S sequence to confirm *Babesia sensu stricto* infection (Table 3). All eight of the raccoons that were coinfecting samples were successfully amplified using the LSU rRNA gene PCR assay. These LSU sequences were (97.3–99.7%) similar to *Babesia lintoni* (Table 3). However, based on sequences from the cox1, 18S rRNA and LSU gene sequences, there were three distinct groups of *Babesia sensu stricto* (Table 3). Unique cox1 and 18S rRNA gene sequences were submitted to GenBank (accession numbers MG986879–MG986888).

Many of the samples positive for the *B. microti*-like sp. using the screen PCR did not produce amplicons with the cox1 PCR protocol, so to confirm infections with this species, we sequenced amplicons from the two different 18S PCR protocols. Near full-length 18S rRNA sequences were acquired for four raccoons and shorter 18S rRNA sequences were obtained for six additional raccoons, including the three raccoons in the 1-week-old litter (Table 2). All of these sequences were identical or most similar (>99.5%) to the *B. microti*-like sp. sequences from raccoons available in GenBank (AB197940, AB935335 and AY144701).

Three tick species were found on young raccoons including *I. texanus*, *I. scapularis* and *D. variabilis*, all from raccoons from Minnesota (Table 4). Damaged ticks that could not be identified to species based on morphological characteristics were identified as *I. texanus* using PCR and sequence analysis. All life stages of *I. texanus* were detected while only larvae and nymphs of *I. scapularis* were found. Infestation with *I. texanus* was first noted on raccoons at 2 weeks of age and the infestation prevalence was similar for all age groups (Fig. 1A). In contrast, *I. scapularis*

Table 1. Prevalence of *Babesia* spp. in young raccoons from Colorado and Minnesota, by age class^a

State	Parasite	Age in weeks no. positive/no. tested (% positive)							Total
		<1 ^b	1	2	3	4	5	6+	
Minnesota	<i>Babesia microti</i> -like sp.	0/3	3/3 (100)	9/19 (47)	13/25 (52)	13/18 (72)	3/5 (60)	9/10 (90)	50/83 (60)
	<i>Babesia sensu stricto</i> group	0/3	0/3	0/19	3/25 (12)	1/18 (6)	1/5 (20)	3/10 (30)	8/83 (10)
	Coinfected	0/3	0/3	0/19	2/25 (8)	0/18	0/5	3/10 (30)	5/83 (6)
Colorado	<i>B. microti</i> -like sp.	NT	3/3 (100)	1/3 (33)	1/3 (33)	NT	3/3 (100)	4/5 (80)	12/17 (70)
	<i>Babesia sensu stricto</i> group	NT	0/3	1/3 (33)	0/3	NT	0/3	2/5 (40)	3/17 (18)
	Coinfected	NT	0/3	1/3 (33)	0/3	NT	0/3	2/5 (40)	3/17 (18)
Total	<i>B. microti</i> -like sp.	0/3	6/6 (100)	10/22 (45)	14/28 (50)	13/18 (72)	6/8 (75)	13/15 (87)	62/100 (62)
	<i>Babesia sensu stricto</i> group	0/3	0/3	1/22 (5)	3/28 (11)	1/18 (6)	1/8 (13)	5/15 (33)	11/100 (11)
	Coinfected	0/3	0/3	1/22 (5)	2/28 (7)	0/18	0/8	5/15 (33)	8/100 (8)

^aAge was not available for six raccoons and were not included in the table.

^bThese three raccoons were near-term and removed *via* caesarian section from a deceased female.

Table 2. Data on *Babesia* infections and ticks on raccoons from identifiable litters from Minnesota.

Litter	Estimated age (weeks)	No. of kits admitted	No. of kits infested with ticks	No. of infected		Species present based on sequence analysis (gene target)
				<i>B. microti</i> -like sp.	<i>Babesia sensu stricto</i> sp.	
1	Fetal	3	0	0	0	ND
2	1.5	5	5 (IT)	3	0	Three with <i>B. microti</i> -like sp. (18S rRNA)
3	1.5–2.5	4	0	2	0	ND
4	1	3	0	3	0	Three <i>B. microti</i> -like sp. (18S rRNA)
5	2	5	0	2	0	ND
6	1.5	2	2 (IT)	1	0	ND
7	2.5–3	5	1 (DV)	4	0	ND
8	4	3	2 (IT)	3	0	ND
9	2	3	1 (DV,IT)	1	0	<i>B. microti</i> -like sp. (18S rRNA)
10	2.5	4	2 (IT)	1	2	Both confirmed <i>Babesia sensu stricto</i> sp. (18S rRNA and LSU) ^a One with <i>B. microti</i> -like sp. (cox1)
11	4–5	2	0	2	2	Both with <i>B. microti</i> -like sp. (18S rRNA); Both with <i>Babesia sensu stricto</i> (LSU) ^b
12	4–5	2	0	2	0	One with <i>B. microti</i> -like sp. (18S rRNA)
13	3	2	0	0	0	ND
14	3	3	3 (DV, IT)	1	0	ND
15	3	3	1 (IT)	1	0	ND
16	4–5.5	3	3 (IT)	2	0	ND
17	3–4	3	1 (IT)	3	0	ND
18	3–4	4	4 (DV, IT)	3	1	ND

IT, *Ixodes texanus*; DV, *Dermacentor variabilis*; Cox1, cytochrome c oxidase subunit I; LSU, large subunit rRNA; ND, not done.

^aRaccoons 16–4482 and 16–4484 in Table 3.

^bRaccoons 16–4596 and 16–4597 in Table 3.

infestations were primarily noted in raccoons older than 5 weeks of age, although a single 3-week-old raccoon was infested with two *I. scapularis* nymphs (Fig. 1A). Infestation prevalence for *D. variabilis* increased with age (Fig. 1A). The mean number of *I. texanus* collected from infested raccoons was highest for raccoons in the 2-week age group with two individuals having 54 and 64 ticks, respectively (Fig. 1B). Other than those two raccoons with high *I. texanus* infestations, tick burdens were generally low with a maximum number of ticks collected from an individual being three *I. scapularis* and 13 *D. variabilis*. Most ticks were found in the ears or on the face (Fig. 2) although two raccoons had ticks present on multiple parts of the body. Presence of ticks was not a significant predictor variable for infection with either piroplasm (*B. microti*-like sp.: $P=0.2393$; *Babesia sensu stricto*: $P=0.3604$).

Using GLM, the only significant variable for raccoons infected with *B. microti*-like sp. was age ($P=0.0059$). According to the GLM, infection of raccoons with *Babesia sensu stricto* was associated with age ($P=0.0017$) and spleen:body weight ratio ($P=0.0005$). Also, raccoons with *Babesia sensu stricto* infections had significantly higher spleen:body weight ratios compared with raccoons infected with *B. microti*-like sp. only or those with no *Babesia* infection ($P=0.0008$ and $P<0.0001$, respectively)

(Fig. 3). Although not significantly different from either group, coinfecting raccoons had increased average spleen:body ratio ($P=0.374$) (Fig. 3).

Discussion

We detected *Babesia* infections in young raccoons from Minnesota and Colorado with the *B. microti*-like sp. detected in individuals as young as 1 week of age. There was a high prevalence of the *B. microti*-like sp. in raccoons from Minnesota and Colorado, and although *Babesia sensu stricto* infections were detected, the prevalence was much lower. We obtained sequence confirmation for the two piroplasms previously reported from raccoons (a *B. microti*-like sp. and *B. lotori*), but we also found possible novel *Babesia* spp. (groups B and C in Table 3). We also noted coinfections occurring in some young raccoons; however, the prevalence of coinfection in both states was much lower than previously reported for adult raccoons in North Carolina, but this is likely due to the lower prevalence of *Babesia sensu stricto* infections among the young raccoons we tested (Birkenheuer et al. 2006). These data extend the known range of *B. lotori* to Minnesota and confirms that *Babesia sensu stricto*

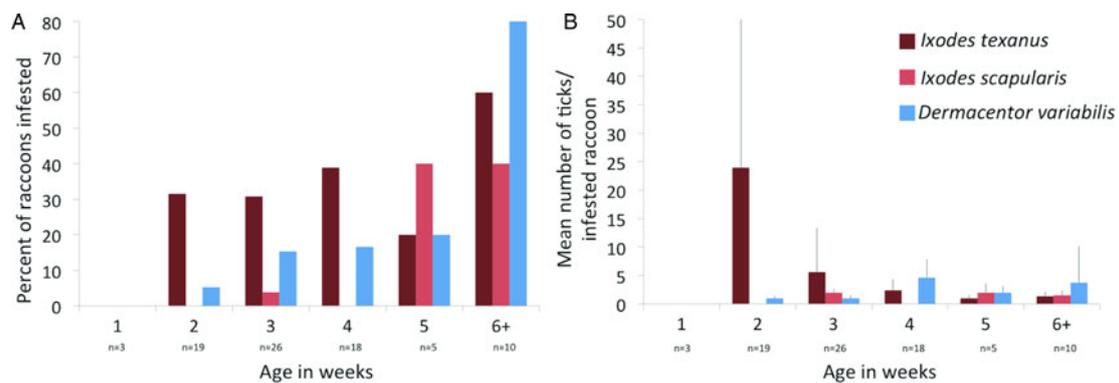
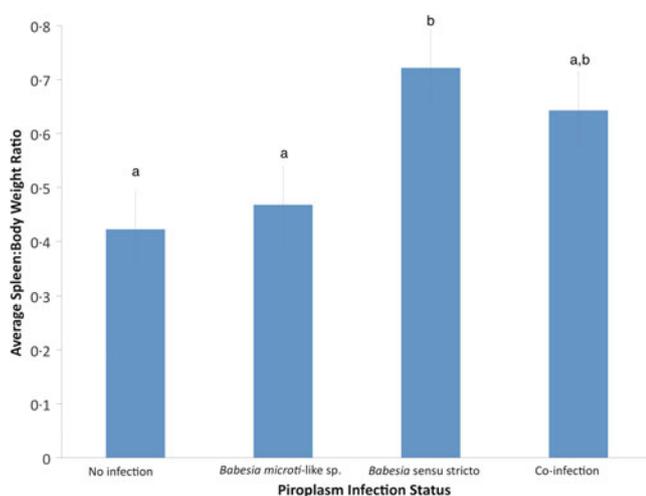
Table 3. Summary of PCR and sequence results for 11 raccoons positive for *Babesia sensu stricto* (eight coinfecting with a *B. microti*-like sp. based on 18S rRNA screening PCR and three raccoons infected with only *Babesia sensu stricto*).

Group	ID	<i>Babesia sensu stricto</i> group ^a	Cytochrome c oxidase subunit 1	18S rRNA gene (long segment)	18S rRNA gene (short segment, screening protocol)	Large subunit rRNA gene
Neonates – coinfecting	16-4596	A	n.a. ^b	99.4% (1013/1019) to <i>Babesia</i> sp. from raccoon (AB197940) – a <i>B. microti</i> -like sp.	99.1% (337/340) to <i>Babesia</i> sp. from Japan (AB935172, AB251608)	98.1% (151/154) to <i>B. lintan</i> (KX698109)
	16-4597	A	n.a.	99.4% (478/481) to <i>Babesia</i> sp. from raccoons (AB197940, AY144701, AB935335) – a <i>B. microti</i> -like sp.	99.4% (326/328) to <i>Babesia</i> sp. from Japan (AB935172, AB251608)	98.1% (151/154) to <i>B. lintan</i> (KX698109)
	16-5713	B	99.8% (863/865) to a <i>Babesia</i> sp. from a maned wolf (KR017881)	n/d ^c	n.a.	99.4% (153/154) to <i>B. lintan</i> (KX698109)
	16-5935	B	90.4% [903/999] to <i>B. vulpes</i> (KC207827) – a <i>B. microti</i> -like sp.	99.5% (551/554) to <i>Babesia</i> sp. from raccoons (AB197940, AY144701, AB935335) – a <i>B. microti</i> -like sp.	98.8% (327/331) to a <i>Babesia</i> sp. from a maned wolf (KR017880)	99.7% (152/154) to <i>B. lintan</i> (KX698109)
	16-5159	C	90.4% [856/949] to <i>B. vulpes</i> (KC207827) – a <i>B. microti</i> -like sp.	98.7% (466/472) to <i>Babesia</i> sp. from raccoons (AB197940, AY144701) – a <i>B. microti</i> -like sp.	n.a.	97.3% (107/110) to <i>B. lintan</i> (KX698109)
	CO-498	B	n.a.	n/d	n.a.	99.4% (153/154) to <i>B. lintan</i> (KX698109)
	CO-1350	B	n.a.	n/d	n.a.	99.4% (153/154) to <i>B. lintan</i> (KX698109)
	CO-861	B	n.a.	n/d	n.a.	99.3% (151/152) to <i>B. lintan</i> (KX698109)
Neonates – only infected with <i>Babesia sensu stricto</i>	16-4484	A	84.2% (758/900) to <i>B. lotori</i> (KR017882)	n/d	n.a.	98.1% (151/154) to <i>B. lintan</i> (KX698109)
	16-5938	A	n/a	n/d	99.1% (338/341) to <i>Babesia</i> sp. from Japan (AB935172, AB251608)	n/d
	16-4482	A	84.2% (758/900) to <i>B. lotori</i> (KR017882)	n/d	99.4% (326/328) to <i>Babesia</i> sp. from Japan (AB935172, AB251608)	n/a

^aThree groups of *Babesia sensu stricto* were identified based on sequences of the three gene targets.^bn.a., not available (PCR either was negative or we were unable to get a clean sequence).^cn/d, not done.

Table 4. Number and stage of ticks collected from young raccoons from Minnesota

Tick species	n	Tick stage			
		Larvae	Nymph	Male	Female
<i>Ixodes texanus</i>	211	7	133	1	70
<i>Ixodes scapularis</i>	9	3	6	0	0
<i>Dermacentor variabilis</i>	49	0	1	18	30

**Fig. 1.** (A) Per cent of young raccoon infested with ticks by age class in weeks (number of raccoons sampled in each age class show below age). (B) Average number of ticks from infested raccoons in each age class in weeks. Number of raccoons sampled is the same as in (A).**Fig. 2.** Ticks on a 1.5-week-old raccoon. (A) An adult *Dermacentor variabilis* on snout. (B) Several nymphal and adult *Ixodes texanus* in an ear. (C) Several adult *I. texanus* in an ear.**Fig. 3.** Effects of *Babesia* infection on average spleen:body weight ratio of young raccoons with standard error bars. Different letters denote significant differences among groups.

spp. occur in Minnesota and the *B. microti*-like sp. occurs in Minnesota and Colorado.

The only previous study to investigate *Babesia* infections in young raccoons was conducted in Connecticut and our data support those findings (Anderson *et al.* 1981). Anderson *et al.* (1981) found that three of four young raccoons were positive for *Babesia* and nymphal *I. texanus* ticks were found on two of the raccoons. However, in the previous study, infections in raccoons were determined based on blood smear analysis and thus the species of *Babesia* present was unknown and the age of the raccoons was not specified. Generally, we found a high prevalence of *Babesia* among identifiable litters and although not all individuals in a litter were infected, these data were similar to data on vertical transmission of *B. microti* in voles (Tolkacz *et al.* 2017).

A primary goal of our study was to determine if young raccoons were infected with *Babesia* and investigate the possible role of vertical transmission as a route of infection. However, because we also detected a high prevalence of tick infestation on raccoons from Minnesota, it is unknown if the infections in young raccoons were acquired vertically from infected female

raccoons or were due to infestation with ticks at a very young age. While we did not find ticks on raccoons younger than 2 weeks of age, our sample size for 1-week-old raccoons was limited so it is possible that raccoons become infested with ticks earlier than we noted. We also did not note any infection in the three fetal raccoons; however, this was also a small sample size and samples from the dam were not available for analysis.

The prepatent period is generally unknown for many piroplasms and varies by transmission route and detection method, so reported data may not be valid for raccoon-infecting piroplasms. For vertical transmission of *B. microti*, voles in Europe had a 3-week prepatent period and experimentally infected BALB/c laboratory mice had a 20-day prepatent period (Bednarska *et al.* 2015; Tolkacz *et al.* 2017). Our data suggest that some *Babesia* infections may be acquired due to vertical transmission, as we detected *B. microti*-like sp. infections in raccoons as young as 1 week old which is generally shorter than prepatent periods associated with tick transmission (e.g., 13–28 days for *B. microti* to rhesus macaques (*Macaca mulatta*) and 13–17 days to BALB/c mice) (Ruebush *et al.* 1981; Li *et al.* 2016).

For *Babesia sensu stricto* spp., short prepatent periods after exposure of hosts to infected ticks have been documented. The prepatent period for *B. canis* transmitted to dogs by *Rhipicephalus sanguineus* was 6 days post-infection, whereas cattle became infected with *Babesia major* within 9–15 days after exposure to infected *Haemaphysalis punctata* (Paraense, 1949; Yin *et al.* 1996). Vertical transmission of *Babesia sensu stricto* sp. has also been noted with beagle puppies, whose mother was intravenously inoculated with *B. gibsoni* prior to mating, showing infection after 14 days (Fukumoto *et al.* 2005). Other cases of vertical transmission of *Babesia sensu stricto* sp. have been reported in puppies infected with *Babesia canis* 6 weeks after birth and in puppies with clinical signs after 8 weeks from presumed vertical transmission of *B. canis canis* (Mierzejewska *et al.* 2014; Adaszek *et al.* 2016). Because infections of raccoons with *Babesia sensu stricto* were not noted until at least 2–3 weeks of age, which is within the time frame of tick-transmitted prepatent periods, it is possible that these two groups of *Babesia*, *B. microti*-like sp. and *Babesia sensu stricto* sp., utilize different transmission strategies.

Ticks, specifically ixodid ticks, are the presumed vectors for *Babesia* sp. and are important to discuss when considering the lifecycle of these raccoon piroplasms (Hunfeld *et al.* 2008). The tick species found on our young raccoons from Minnesota are commonly reported on raccoons (Dennis *et al.* 1994; Ouellette *et al.* 1997; Hersh *et al.* 2012). *Ixodes texanus* was the most common and abundant tick found on the raccoons which was expected as this species is found on host's year-around and is assumed to be acquired within the nests of their vertebrate hosts (Sonenshine, 1993; Dharmarajan *et al.* 2016). This tick species has a widespread distribution in the USA (Eastern and Midwestern USA, California, and Alaska and likely many states in between) (Ouellette *et al.* 1997; Gabriel *et al.* 2009; Durden *et al.* 2016). The other two species found on our raccoons included *D. variabilis* (restricted to the Eastern USA and in isolated populations in California) and *I. scapularis* (restricted to the Eastern USA) (Bishopp and Trembley, 1945). Larval and nymphal stages of *I. scapularis* feed on a wide range of small to medium-sized hosts (mammals, birds, lizards), including raccoons, and this species is an important vector of *Borrelia burgdorferi*, the causative agent of Lyme disease, and *B. microti*, the primary causative agent of human babesiosis in the USA (Hersh *et al.* 2012). In our study, only larvae and nymphs of *I. scapularis* were found on raccoons and in very low numbers, most likely because of the earlier seasonal activity of these stages compared with adults, which are more often found on hosts in fall

and winter (Bishopp and Trembley, 1945). Raccoons younger than 6–8 weeks most likely acquire ticks from the mother, as young of this age do not typically venture from the nest, while older individuals (7–10-week-old young) are more active and can become infested with ticks outside of the nest (Schneider *et al.* 1971; Gehrt and Fritzell, 1998).

Piroplasm infections in most species of wildlife are considered to be of low pathogenicity, although under certain circumstances (e.g., coinfections, immunosuppression, stress, climate factors, etc.) they may cause disease (Penzhorn, 2006; Yabsley and Shock, 2012). Examples include babesiosis in African lions suffering from a concurrent canine distemper virus outbreak and decreased food availability, and the development of fatal babesiosis in black rhinoceros due to the stress of capture for translocation efforts (Penzhorn, 2006; Munson *et al.* 2008). In raccoons, *Babesia* infections are presumed to be of little clinical significance but most reports are surveys of healthy free-ranging adults. One possible clinical case in a raccoon was a single juvenile raccoon from Illinois that was infected with *B. lotori* (Birkenheuer *et al.* 2006). The raccoon was found non-ambulatory with pronounced anaemia, hypoproteinaemia, hypalbuminaemia and elevated alanine aminotransferase with rare intraerythrocytic *Babesia* parasites. It was treated for *Babesia* and released; however, it is unknown if it was the *Babesia* infection that caused the clinical signs or if they were the results of a secondary infection (Birkenheuer *et al.* 2006). In general, clinical disease is likely to be more pronounced in young animals. Studies on vertical transmission of piroplasms in several hosts indicate that clinical signs in infected young generally occur between 17 and 25 days (Fukumoto *et al.* 2005; Bednarska *et al.* 2015; Brown *et al.* 2015; Adaszek *et al.* 2016). Because our sampled animals were not available for antemortem testing, we used the ratio of spleen weight:body weight as a measure of possible disease. The association with splenomegaly and *Babesia sensu stricto* infections, but not with *B. microti*-like sp. infections, suggests that early infections with *B. lotori* or the possible novel *Babesia* sp. may cause clinical disease in young raccoons. Unfortunately, because these raccoons were dead on arrival or euthanized on entry, no clinical pathology data were collected nor was any histologic analysis done to determine the cause of death or illness (although many were admitted because they were orphaned, not because they were sick). Splenomegaly is one of many common findings of clinical babesiosis in many host species (Kawabuchi *et al.* 2005; Mierzejewska *et al.* 2014; Solano-Gallego *et al.* 2016), including puppies that acquired *B. canis* infection through vertical transmission (Mierzejewska *et al.* 2014). *Babesia* has been detected in raccoons with splenomegaly in Japan; however, only raccoons with splenomegaly were tested and the prevalence of *Babesia* was low, possibly because raccoons were introduced to Japan (Kawabuchi *et al.* 2005; Jinnai *et al.* 2009). It is possible that *Babesia* spp. of certain wildlife may be more pathogenic than currently recognized but only impact very young animals that are rarely studied.

Because we obtained *B. microti* sequences with the cox1 PCR, this protocol can amplify both *Babesia sensu stricto* species and *B. microti*-like sp. Unfortunately, due to financial constraints, cloning of these coinfecting samples was not possible for this study. However, there were no polymorphic bases present in the cox1 sequences although the sequences that failed may have been due to mixed amplicons. We did not PCR test ticks collected from raccoons for piroplasms because ticks were all potentially blood-fed so any positives could have occurred from ingestion of infected blood from the raccoon, the previous infection from another raccoon, or vertical transmission of piroplasms within infected ticks. One of our objectives was to investigate the possibility of vertical transmission of piroplasms to raccoons but since we detected ticks at a young age and dams were not available for

testing, we could not determine if transmission of piroplasms occurred vertically or *via* tick transmission.

In summary, we show that several species of *Babesia* infect very young raccoons. In addition, we showed that several species of ticks are parasitizing these young raccoons, so it is currently unknown if these infections are a result of vertical transmission or tick-transmission due to early infestation. Finally, we note that young raccoons infected with *Babesia sensu stricto* spp. have a higher spleen:body weight ratio suggesting possible clinical disease associated with infection. Additional studies are needed to better understand the natural history, diversity and impact of *Babesia* infections in raccoons.

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Conflict of interest

None.

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. Although no animals were euthanized for the purposes of this study, the collection of biological samples for pathogen testing was reviewed and approved by UGA's Institutional Animal Care and Use Committee (A2014 10-018).

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