PRESENCE, DISPERSAL, AND GEOGRAPHIC DISTRIBUTION OF *BABESIA* PIROPLASMS IN RACCOONS (*PROCYON LOTOR*) IN THE UNITED STATES AND CANADA

By

KAYLA BUCK GARRETT

(Under the Direction of Michael Yabsley)

ABSTRACT

Babesia are intraerythrocytic protozoans, many of which have veterinary/medical importance. Two morphologically similar species, *Babesia lotori* and *Babesia microti*-like sp., occur in raccoons (*Procyon lotor*). Little is known of the distribution and prevalence of these parasites and recently maned wolves (*Chrysocyon brachyurus*) in a Missouri zoo have been diagnosed with severe/fatal babesiosis caused by *B. lotori*. Raccoon blood and spleen samples were obtained from locations in the USA and Canada and tested using species-specific PCR assays. *B. lotori* prevalence was highest in the Southeast (20-45% [142/519]) and *B. microti*-like sp. was detected at all sites with general prevalence highest in the Southeast (67-100% [382/519]). Coinfections were common. For neonate raccoons, 62% (66/106) were positive for *B. microti*-like sp., 10% (11/106) for *B.* sensu stricto, and 7.5% (8/106) were co-infected. Currently transmission route is unknown for both *Babesia* spp., thus these data may assist in determining potential vector(s).

INDEX WORDS: *Babesia*, raccoons, ticks, vertical transmission, geographic distribution

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DEDICATION

I would like to dedicate this to my parents, Chris and Mary Buck, both of whom provided me with not only financial assistance, but also love, support, and many answered phone calls. Thanks for still loving me, even though I got rabies once or twice during my research.

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I first want to thank my husband, Josh Garrett, for always being there for me when I didn't think I had anything left in me, and for staying in Athens with me to help me follow my dream. And thank you for listening to every presentation that I needed to practice, even when the research hardly changed. I'm fairly certain you are an expert of *Babesia* now, and I would not have succeeded if it wasn't for your love and support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

The northern raccoon (*Procyon lotor*) is a native, medium-sized omnivore found throughout much of the United States and Canada, as well as several European and Asian countries where introduced populations have been established. Raccoons utilized a wide range of habitats including urban and suburban areas, allowing them to easily become nuisance species. This results in an increased contact between raccoons and humans, domestic, and captive/zoo animals, which can lead to an increased risk or concern about diseases. Pathogens such as rabies virus, canine distemper virus, *Baylisascaris procyonis*, and more recently *Babesia* species have been a reason for much concern with raccoons. This study aims to increase our knowledge of *Babesia* species in raccoons, which to date are poorly studied.

The specific objectives of this study are:

1. To investigate the prevalence, distribution, and diversity of *Babesia* spp. in raccoons in selected areas of the United States and Canada.
    i. Hypothesis: There will be a different distribution of the two known *Babesia* species in raccoons which is likely related to the distribution of suspected or possible vector(s).
2. To examine the intra-specific variation of the *Babesia microti*-like sp. and *Babesia lotori* in raccoons in different geographic regions of North America.

i. Hypothesis: Because raccoon have historically occurred throughout North America, we expect to see intraspecific genetic variations for *Babesia* spp. between different geographic regions.

3. To determine if, and at what age, infections can occur in young and neonate raccoons and what tick species may infest young raccoons.
    i. Hypothesis: Raccoons become infected at an early age, either through vertical transmission or via ticks; likely a nest dwelling species of tick such as *I. texanus.*

LITERATURE REVIEW

Background

Babesia spp. are obligate intraerythrocytic protozoan parasites and are one of the most common haemoparasites in the world (Uilenberg, 2006; Hunfeld et al., 2008; Lack et al., 2012; Schnittger et al., 2012; Yabsley and Shock, 2013). This group of parasites was first discovered by Babes (1888), who observed these parasites in erythrocytes in cattle in Romania that suffered from 'red water fever'. Later, a cattle parasite named *Pyrosoma bigeminum* was found in the US by Smith and Kilborne (1893), and that the parasite was tick transmitted. *P. bigeminum*, later named *B. bigemina,* was the first species of parasite with confirmed transmission by an arthropod. Later that same year, Starcovici (1893) reclassified these piroplasm parasites as a new genus, *Babesia,* named in honor of Victor Babes (Telford 3rd et al., 1993; Uilenberg, 2006; Lack et al., 2012; Schnittger et al., 2012).

Babesia spp. are classified in the phylum Apicomplexa, class Piroplasmea, order Piroplasmida, and family Babesiidae. The order Piroplasmida contains multiple genera of tick-borne pathogens within 3-4 families, including Babesiidae, Theileriidae, Dactylosomatidae (parasites of fish and amphibians) and a fourth family, Anthesomatidae (parasites of spiny mice [*Acomys* spp.], which is sometimes included in the Dactylosomatidae). Some of the most important pathogens of mammals are included in the genera *Theileria* and *Cytauxzoon* in the family Theileriidae, and *Babesia* in the family Babesiidae (Telford 3rd et al., 1993; Hunfeld et al., 2008; Lack et al., 2012; Schnittger et al., 2012). These three genera can be distinguished by several features: *Babesia* spp. only infect red blood cells and lack schizont formation, whereas *Theileria* has schozogony in lymphocytes and *Cytauxzoon* has schizogony in mononuclear phagocytic cells (Uilenberg, 2006; Hunfeld et al., 2008; Lack et al., 2012). In addition, some *Babesia* spp. can be transmitted transovarily by ticks, while *Theileria* and *Cytauxzoon* can only be transmitted transstadially in ticks (Figure 1.1) (Uilenberg, 2006). The *B. microti* group has at least one member that has exoerythrocytic schizont formation, suggesting a closer affinity to the Theileriidae; however, phylogenetic analyses consistently place this group basal to all of the piroplasms. Another group of piroplasms, discussed in more detail later, is the western piroplasms/western *Babesia*, and includes *B. conradae* as shown in Figure 1.1. Currently the typical characteristics used to classify the parasites into Theileriidae or Babesiidae are not known for the western group, such as if it is transmitted transovarilly or just transtadially in ticks or if it infects more than just erythrocytes.

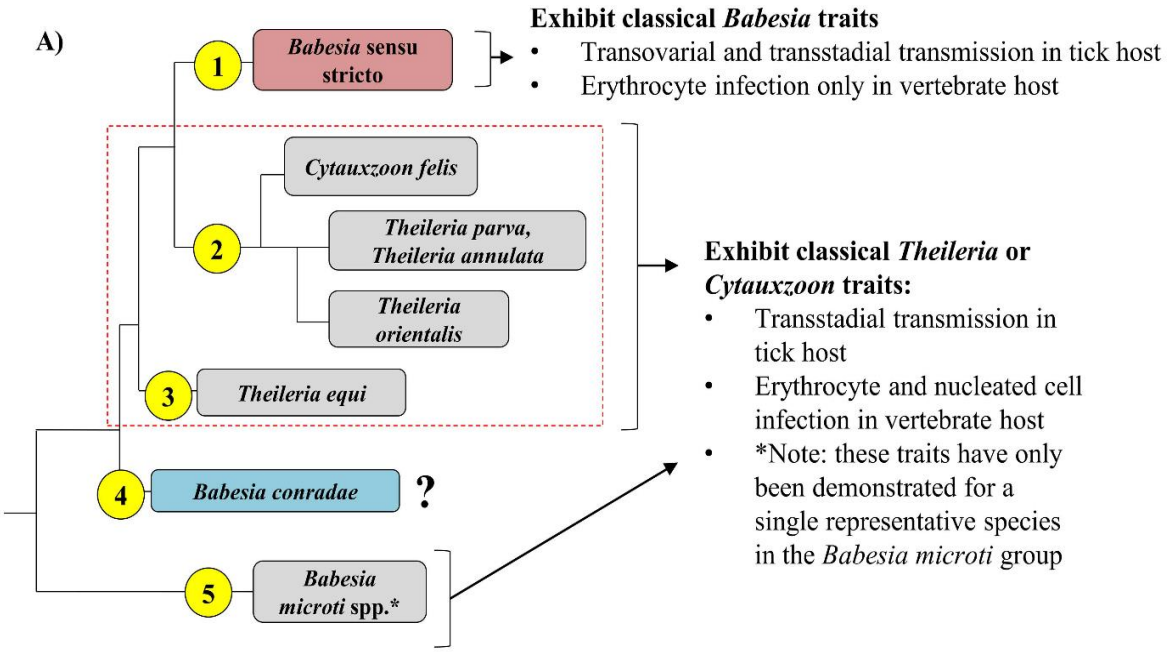


FIGURE 1.1. Various biological characteristics of the five groups of piroplasms. From Schreeg et al. 2016

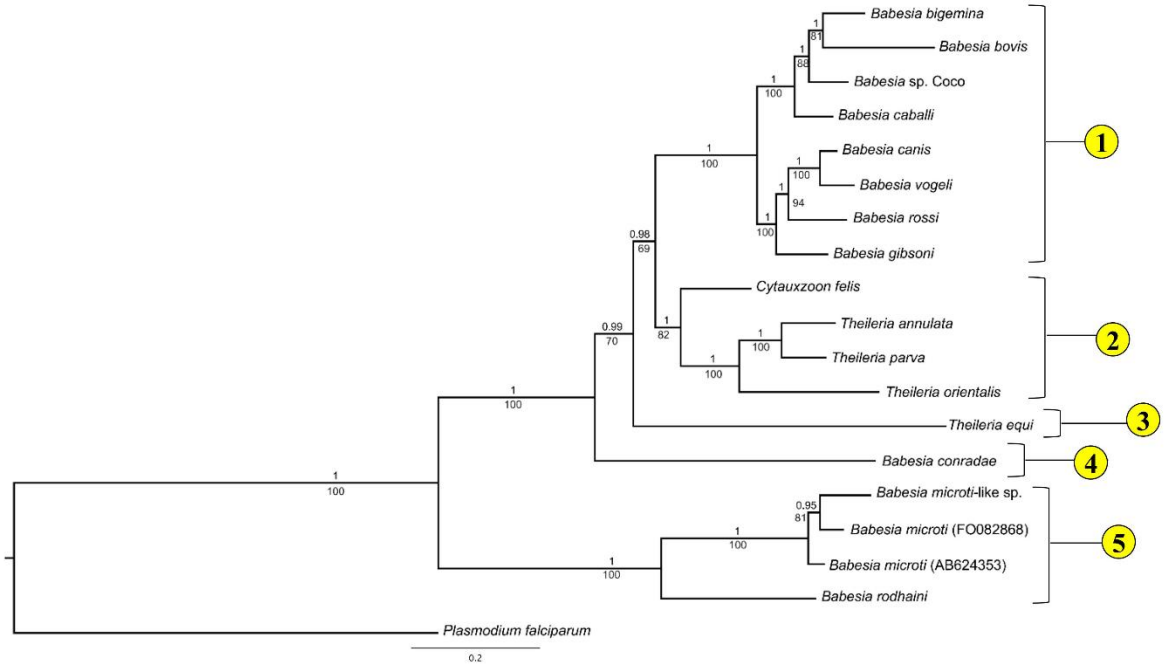
Babesia is an incredibly diverse genera with over 100 species having been identified in mammals and birds, and the number of recognized species increases annually, even if you only consider the *Babesia* sensu stricto (group 1 in Figure 1.1). Most *Babesia* spp. infect wildlife and many of these are either not associated with clinical disease or disease is rare; however, there are many species that have major impacts in domestic and livestock animals, humans, and some wildlife species. Historically it was presumed there was high host specificity for these piroplasm parasites, but many species are currently recognized in multiple host species (Birkenheuer et al., 2006; Hunfeld et al., 2008; Lack et al., 2012; Schnittger et al., 2012; Yablsey and Shock, 2013).

Babesia species were classically identified based on the size and shape of the trophozoites in the erythrocytes, the number of merozoites, and the host origin. The terms "piroplasma" or "piroplasms" are used for the organisms in Piroplasmida and are derived from

the “pear-shape” many of the organisms develop (Uilenberg, 2006). Other morphologic forms of *Babesia* species include rings, tetrads of pear-shaped organisms, and amoeboid-shapes. Based on general size, *Babesia* were separated into two groups, piroplasms smaller than 3µm, and those larger than 3 µm (Telford 3rd et al., 1993; Hunfeld et al., 2008; Yabsley and Shock, 2013).

Recently, genetic characterization of *Babesia* spp. has been used to confirm species identification, to identify cryptic species, and to investigate phylogenetic relationships. In addition, this is a useful tool as some *Babesia* species may have variable morphologic characteristics in different hosts, while others can have multiple morphologic characteristics while in the same host (Scholtens et al., 1968; Anderson et al., 1981; Zintl et al., 2003). Although these molecular characterization studies have helped clarify relationships, they have also created controversy. The most important is the finding that *B. microti* and related parasites, and the western piroplasms are not phylogenetically related to other parasites classified in the *Babesia* genus. Based on multi-gene analyses there are 5 well-accepted groups: the *B. microti*-like group, western piroplasms group (with some species in the group named in genus *Babesia*), theilerids (*Theileria/Cytauxzoon* spp.), *Theileria equi*, and the *Babesia* sensu stricto group. Importantly, the group of parasites that are called the western piroplasms (or prototheilerids by some researchers) is well supported in many analyses as a distinct group, but the exact phylogenetic relationship of this group of parasites to the other genera is poorly understood (Figures 1.2 and 1.3) (Criado-Fornelio et al., 2003; Yabsley and Shock, 2013; Shreeg et al., 2016).

A) Concatenated mitochondrial and 18S nucleotide sequence



B) Five distinct *Piroplasmida* groups

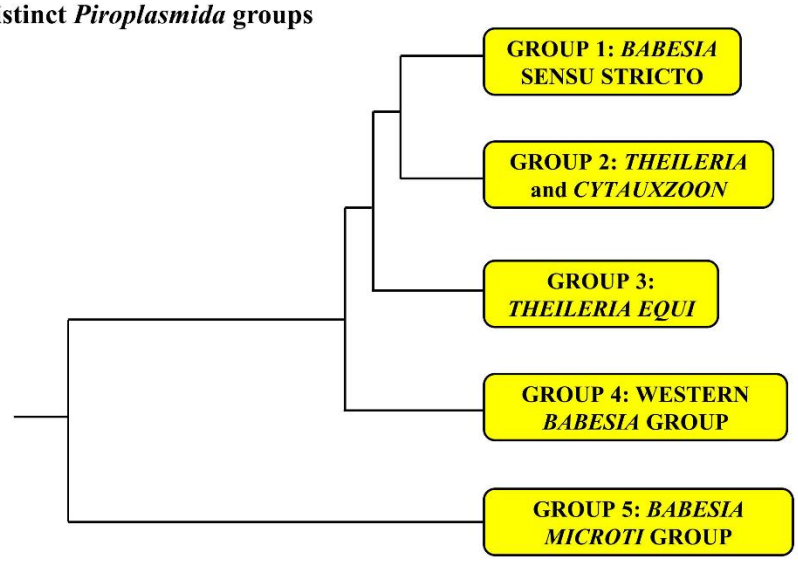


FIGURE 1.2. Phylogenetic trees illustrating the five primary groups of piroplasm parasites. A) Tree showing various example species included in these groups based on analysis of 18S rRNA and full mitochondrial gene sequences. B) The names of the five groups. From Schreeg et al., 2016.

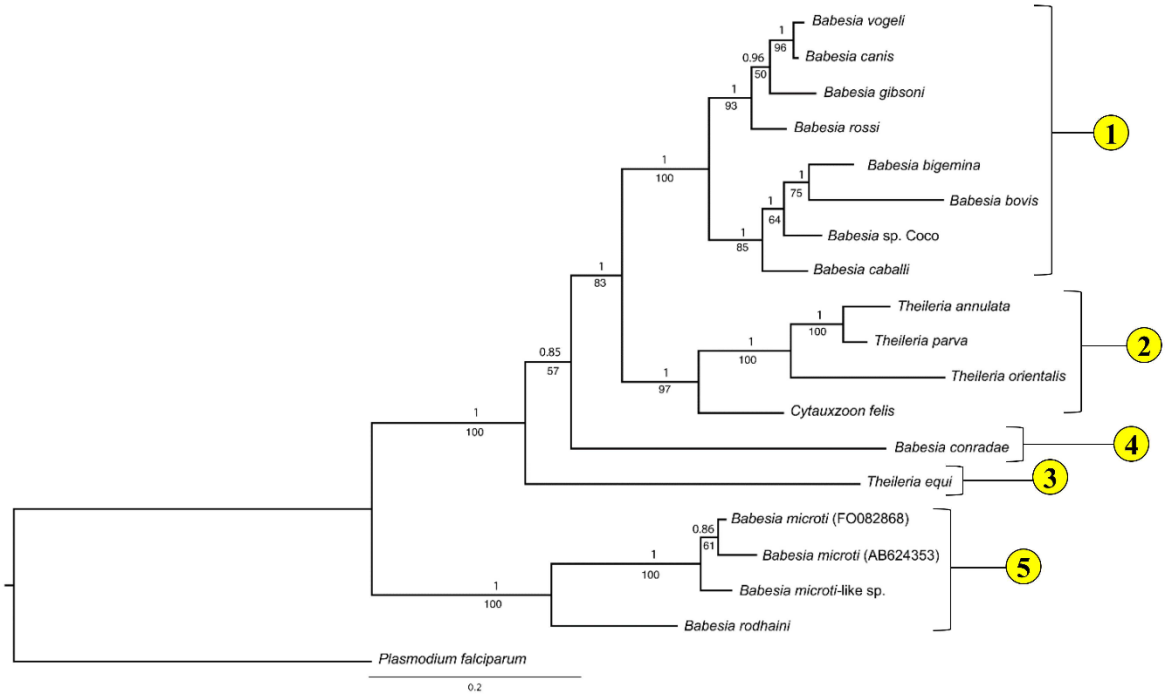


FIGURE 1.3. Phylogenetic analysis of cox1 amino acid sequences is sufficient to recognize the same five piroplasm groups as detected using concatenated mitochondrial and 18S nucleotide sequences. From Schreeg et al., 2016.

The *B. microti*-like group is an important group and is also a group in taxonomic flux. The members of the *Babesia microti*-like group are genetically similar but distinct from *B. microti* that infects rodents and humans. Thus, researchers refer to these parasites as *B. microti*-like sp. from raccoons (*Procyon lotor*), skunks (*Mephitis mephitis*), river otters (*Lontra canadensis*), etc., based on host origin (i.e. *B. microti*-like sp. raccoon, *B. microti*-liks sp. skunk) (Goethert and Telford, 2003; Birkenheuer et al., 2007; Schnittger et al., 2012; Yabsley and Shock, 2013; Shreeg et al., 2016). This has led to considerable confusion when attempting to refer to a specific species.

Regardless, this group is distinct from the *Babesia* sensu stricto group and many researchers have suggested they be moved to another genus; however, there seems to have been hesitation on renaming the genus as it currently contains a human pathogen, *Babesia microti* (Nakajima et al., 2008). Humans can experience babesiosis, but if the name of the pathogen is changed the medical community would have to adopt another name. This can often be a difficult transition such as when the human granulocytic ehrlichiosis agent was reclassified as an *Anaplasma* sp. and could no longer be called ehrlichiosis (Chen et al., 1994; Dumler et al., 2001; Dumler et al., 2005).

These *B. microti* and *B. microti*-like species have been classified into multiple clades but can be collapsed into two major clades. Clade 1 includes rodent parasites and zoonotic *Babesia microti*, while Clade 2 includes numerous parasites from various carnivore species (Figure 1.4) (Goethert and Telford 3rd, 2003; Hunfeld et al., 2008; Schnittger et al., 2012; Shreeg et al., 2016). Recently there has been some attempt to help with the taxonomic confusion by naming one of the parasites in this group (Baneth et al., 2015). The canine and fox parasite, now known as *B. vulpes,* had previously been published as canine *B. microti*, a *B. microti*-like sp., Spanish dog isolate, *Theileria annae*, *Babesia annae* and simply *Babesia* sp., which are now all considered synonyms. Still, this move was criticized by some who believe this contributes to this group's taxonomic confusion and they should just be referred to as the "microti group" (Harris, 2016).

FIGURE 1.4. Phylogentic tree of *B. microti* and *B. microti*-like sp. from carnivores. The *B. microti*-like spp. from carnivores clearly are distinct from rodent and primate infecting *B. microti*. From Baneth et al., 2015.

Lifecycle

The general lifecycle of *Babesia* piroplasms begins in an infected tick host where sexual reproduction of the parasite occurs. After the sporozoites mature, which usually takes a couple days, they become infective and the tick is able to transmit the pathogen (Uilenberg, 2006; Phair et al., 2012). The piroplasms are then transmitted to other hosts via injection into a host during the tick's feeding (Uilenberg, 2006; Hundfeld et al., 2008; Lack et al., 2012; Schnittger et al., 2012; Solano-Gallego et al., 2016). Replication occurs within the erythrocytes of the host where two to four daughter cells are created that will then infect other erythrocytes (Uilenberg, 2006; Hunfeld et al., 2008; Lack et al., 2012; Schnittger et al., 2012). Many *Babesia* infections can become chronic and the cycle continues when a tick becomes infected during a blood meal (Uilenberg, 2006; Hunfeld et al., 2008; Hersh et al., 2012).

Babesia *infections in humans*

Babesia microti is the primary cause of babesiosis in humans in the United States and was first detected in 1969 in a California patient (Scholtens et al., 1968). *B. microti* is maintained in rodent reservoirs and is transmitted by various *Ixodes* spp., primarily *I. scapularis* in the Eastern US and *I. pacificus* in the western US. Based on genetic characterization, *B. microti* is a large species complex with some strains being zoonotic while others only seem to utilize rodents as hosts. The mortality rate is fairly low for *B. microti* infections in people (~5%) and many individuals do not even know they are infected (Meldrum et al., 1992; Goethert and Telford 3rd, 2003; Binda, 2016). Most cases of clinical babesiosis occur in immunocompromised, young, old, or splenectomized individuals. Although most human infections are acquired through a tick bite, the parasite can also be transmitted through blood transfusion and from mother to child (Hunfeld

et al., 2008; Young et al., 2012; Binda, 2016). In fact, in North America, *B. microti* is the most common blood transfusion-transmitted disease (Young et al., 2012). Typical disease associated with *B. microti* infections in humans can range vastly, with some individuals having no illness, and others having mild, flu-like symptoms such as malaise, fever, chills, body aches, nausea, rash, and occasionally splenomegaly, with most symptoms lasting anywhere between one to nine weeks. Severe disease from *B. microti* typically manifests if the individual is splenectomized or immunocompromised and results in malarial-like symptoms, such as high fever, jaundice, or severe anemia that can lead to mortality (Yabsley and Shock, 2012; Binda, 2016).

Babesia divergens is the main cause of babesiosis in Europe with many untreated cases being fatal after only three weeks (Hunfeld et al., 2008; Yabsley and Shock, 2012). *Babesia divergens* presents with hemoglobinuria symptoms similar to malaria, and organ failure generally being the cause of death (Telford 3rd et al., 1993; Yabsley and Shock, 2012). In humans, severe *Babesia* infections are typically treated with antiparasiticides (usually clindamycin), transfusions (blood must be screened for presence of possible *Babesia* piroplasms), or dialysis (Gray et al., 2010; Binda, 2016).

Although *B. microti* and *B. divergens* account for the vast majority of *Babesia* infections in humans, there are several other species of *Babesia* detected humans from North America, Europe, and Asia including *B. venatorum, B. duncani, B. divergens*-like (MO1-type), *B.* sp. TN, *B.* sp. KO1 and *B.* sp. (ovine) (Herwaldt et al., 1996; Kjemtrup and Conrad, 2000; Herwaldt et al., 2003; Conrad et al., 2006; Kim et al., 2007; Schnittger et al., 2012).

Importance of Babesia *to livestock and domestic pets*

Babesia was first discovered in cattle by Babes in 1888 and has since been a cause of major diseases in livestock. Two species, *B. bovis* and *B. bigemina*, can cause red water fever in cattle which can lead to decreased milk and meat production, abortions, and mortality. Discovery of infection can lead to cattle trade restrictions, making it one of the most economically important arthropod-transmitted diseases in cattle (Bock et al., 2008; Schnittger et al., 2012). The disease was eradicated from the USA, but elsewhere outbreaks still occur and it remains a globally important disease. Eradication of the disease in the USA is estimated to save three billion dollars per year (Schnittger et al., 2012). In Europe, another parasite, *B. divergens*, is not only a cause of human babesiosis but can also infect and cause disease in cattle. In horses, *Babesia caballi* can cause a chronic infection that reduces performance, thus making it a concern for the horse racing industry (Schnittger et al., 2012). In parts of Europe, Africa and Asia, *Babesia* spp. are important pathogens of sheep and goats (Uilenberg et al., 1980; Hasherni-Fesharki and Uilenberg, 1981; Lewis et al., 1981; Friedhoff, 1997; Bai et al., 2002; Schnittger et al., 2003).

Domestic dogs can be infected with multiple species of *Babesia* including *B. canis volgeli, B. canis canis, B. canis rossi, B. conradae, B. gibsoni*, and *B.* sp. Coco. Some species are highly pathogenic (*B. rossi*), while others are generally only subclinical or are not greatly pathogenic; however, even *B. vogeli, B. canis, B. conradae, B. gibsoni* can occasionally cause severe babesiosis and death (Birkenheuer et al., 2003; Schnittger et al., 2012; Solano-Gallego et al., 2016). *Babesia* infections have also been reported in domestic cats; however, typically the cats remain asymptomatic with possible mild clinical disease expressed as anemia and jaundice.

The proposed species found in domestic cats in Europe is a subspecies of *Babesia canis*, termed *Babesia canis subsp. presentii* (Baneth et al., 2004; Solano-Gallego and Baneth, 2010). Other *Babesia* species in cats include *Babesia felis*, typically in South African cats, and *Babesia cati* in cats in India (Solano-Gallego and Baneth, 2011).

Unlike domestic animals, wildlife species often harbor *Babesia* infections with no severe clinical signs; however, they can develop disease for many reasons including becoming highly stressed or malnourished, exposure to high numbers of infected ticks after living in a tick free environment, becoming infected with an unnatural *Babesia* spp., or coinfection with other pathogens (Penzhorn, 2006; Munson et al., 2008; Schnittger et al., 2012). Some examples of this include black rhinoceroses that were infected with *Babesia bicornis*, but didn't experience any disease until after stressful relocation efforts. Additionally, lions infected with *B. leo* do not generally experience disease; however, if under stress or when co-infectioned with canine distemper virus, severe and even fatal disease can develop (Penzhorn, 2006; Munson et al., 2008).

Raccoons and Babesia *infections*

Raccoons (*Procyon lotor*) are medium sized omnivores that are native to the United States and Central America (Figure 1.5). Due to legal and illegal movements, invasive populations of raccoons have established in parts of Europe as well as Asia, such as Japan (Sherman, 1954; Aliev and Sanderson, 1966; Ikeda et al., 2004; Dusher et al., 2017). Raccoons are highly adaptable and can utilize a wide range of habitats including, but not limited to, hardwood forests, agricultural lands, and other numerous anthropogenic-modified landscapes, such as urban and suburban areas (Hygnstrom et al., 1994; Prange et al., 2003; Ikeda et al.,

2004). In many anthropogenically-adapted landscapes raccoons have become a nuisance species due to the damage they can cause as well as the pathogens they are capable of harboring and spreading. In the eastern US, raccoons are the primary reservoir for rabies and are also hosts for *Baylisascaris procyonis,* an important zoonotic parasite.

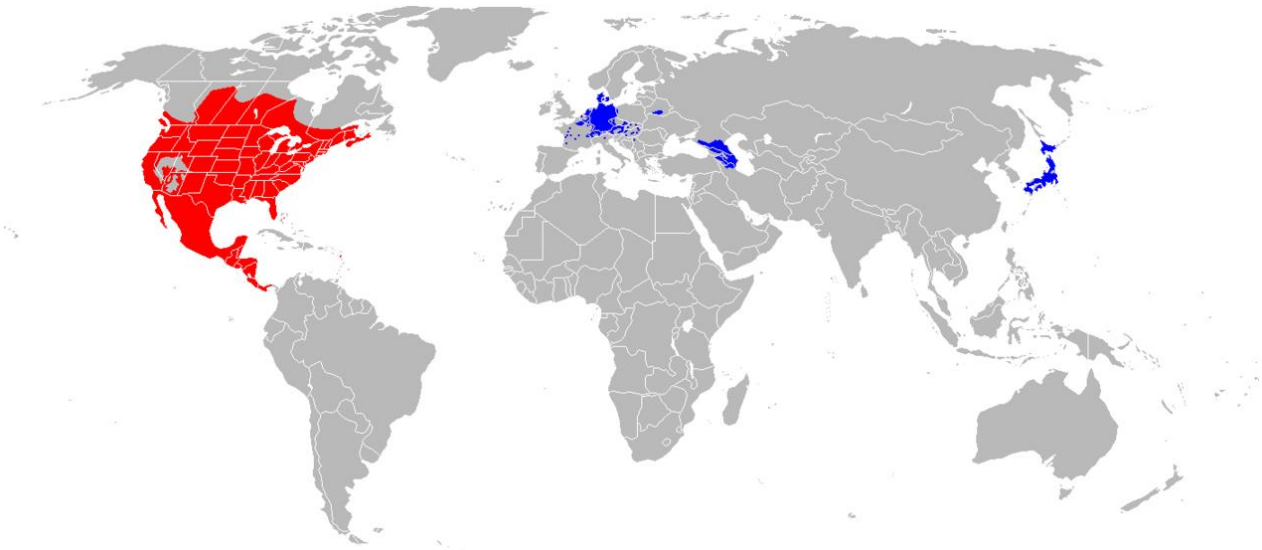


FIGURE 1.5. Approximate distribution of raccoons in their native range (red) and introduced range (blue). However, note that raccoon populations have been now confirmed in many other countries in Europe (e.g., Spain) and Asia (e.g., China). (Figure from Wikipedia)

The prevalence and diversity of *Babesia* in procyonids is poorly understood. Since first reported in a raccoon at the Zoological Gardens of London, England in 1926, there have been numerous reports of small piroplasms (<3 µm) in raccoons; however, few of these were genetically classified (Wenyon and Scott, 1926; Shaffer et al., 1978; Frerichs and Holbrook, 1970; Anderson et al., 1981; Birkenheuer et al., 2006; Birkenheuer et al., 2007). All of the piroplasms that have been reported in raccoons are morphologically similar, but based on sequence analysis there are two distinct species in the United States: *Babesia lotori* (in the sensu

stricto clade) and a *Babesia microti*-like sp. (in the *microti*-like in carnivores clade) (Frerich's and Holbrook, 1970; Anderson et al., 1981; Goethert and Telford, 2003; Birkenheuer et al., 2007). The *B. microti*-like sp. has also been reported in Japan as well as two different lineages of *Babesia* sensu stricto parasites (Jinnai et al., 2009). One of these lineages in related to *B. lotori* and a parasite reported from a Japanese Ixodes tick (in group 2 in Figure 1.6) whereas the other group was in a distinct clade (group 1 in Figure 1.6).

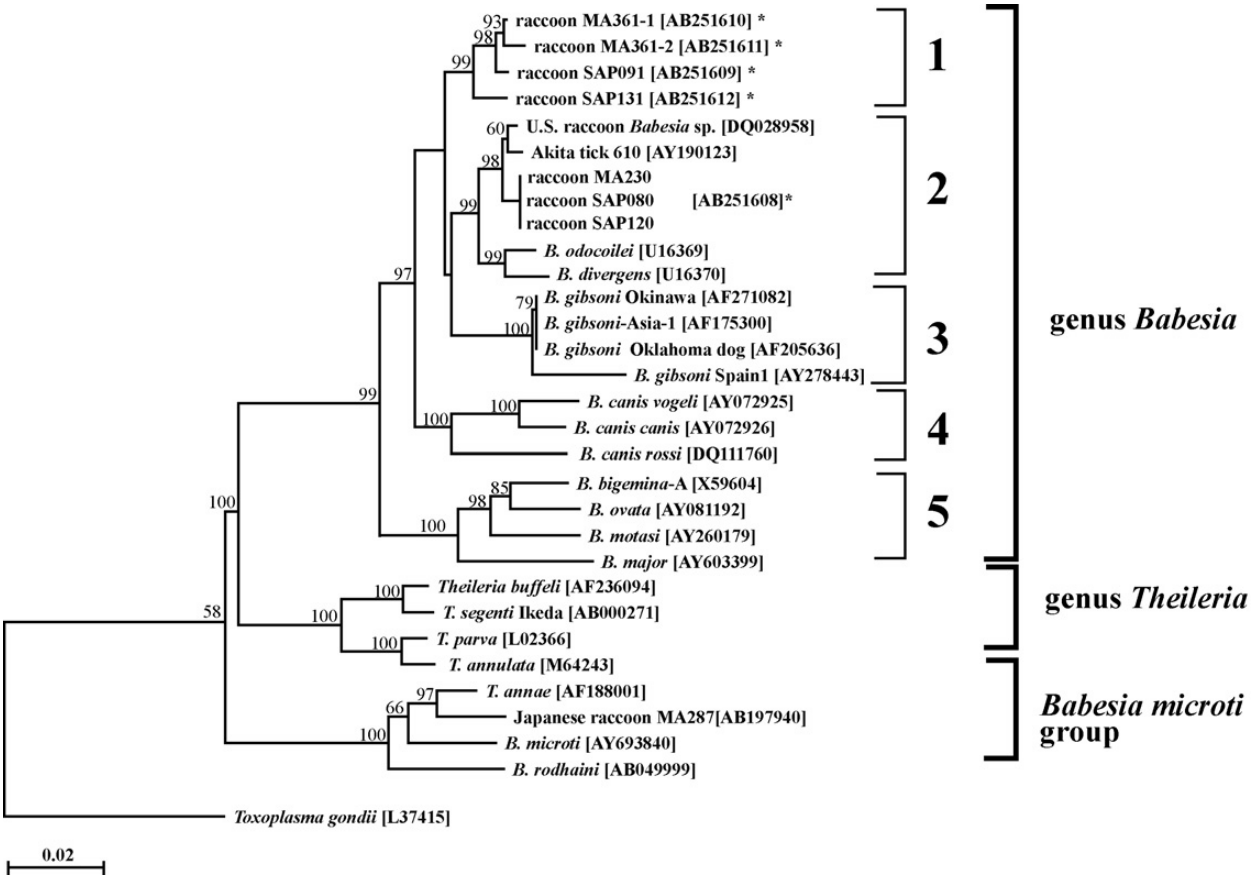


FIGURE 1.6. Phylogenetic tree showing the three groups of *Babesia* detected in Japanese raccoons (in Groups 1, 2, and the *B. microti*-group). From Jinnai et al., 2009.

The nomenclature of raccoon *Babesia* piroplasms is historically complicated and inconsistent because *Babesia* in raccoons was initially referred to as *Babesia procyoni* or *Babesia procyonis* (Ristic and Lewis, 1977; Shaffer et al., 1978; Frerichs and Holbrook, 1970; Levine, 1971; Anderson et al., 1981). However, a large *Babesia* sp. (>3 µm) previously described from raccoon dogs (*Nyctereutes procyonoides*) was named *Piroplasma procyoni* which became *B. procyoni* when the genus *Piroplasma* was reclassified as *Babesia* (Anderson et al., 1981; Birkenheuer et al., 2006). Because of this, Anderson et al. (1981) established the name *B. lotori* for small piroplasms in raccoons from North America to distiguish this parasite from *B. procyoni* in raccoon dogs. During a study of *B. microti* in various hosts from Massachusetts, a second species of raccoon *Babesia* was dicovered and this *Babesia* species was closely related to *Babesia microti* that infects humans and rodents (Goethert and Telford, 2003). This *Babesia microti*-like species found in raccoons currently has no official nomeclature and thus results in confusion with the other *B. microti*-like sp. in other carnivore hosts (Goethert and Telford, 2003; Birkenheuer et al., 2006; Birkenheuer et al., 2007). Currently there are two species of *Babesia* known in raccoons from the United States, *B. lotori* and *B. microti*-like sp., and co-infections with these two parasites were common in raccoons from North Carolina (Birkenheuer et al., 2007).

Identification of Babesia *infections*

As noted earlier, historically *Babesia* spp. were identified morphologically using a Gimesa blood stain and viewing red blood cells for the presence of piroplasms. However, for *Babesia* species in raccoons this method not sufficient because: 1) molecular detection is significantly more sensitive and 2) the two known *Babesia* species in raccoons in the US and

Japan are morphologically similar (i.e., <3µm with multiple different shapes including: round, uni-nucleated “rings”, amoeboid, piriform and paired piriforms) (Figures 1.7-1.9) (Anderson et al., 1981,Telford and Forrester, 1991; Birkenheuer et al., 2006; Phair et al., 2012). Thus, for species identification, PCR (polymerase chain reaction) and sequence analysis are required. Unfortunately, the use of PCR assays for identification of *Babesia* infections has only recently been implemented and therefore historical studies that report *Babesia* could represent either parasite species. This leaves much uncertainty about the natural history of these two *Babesia* spp. in raccoons.

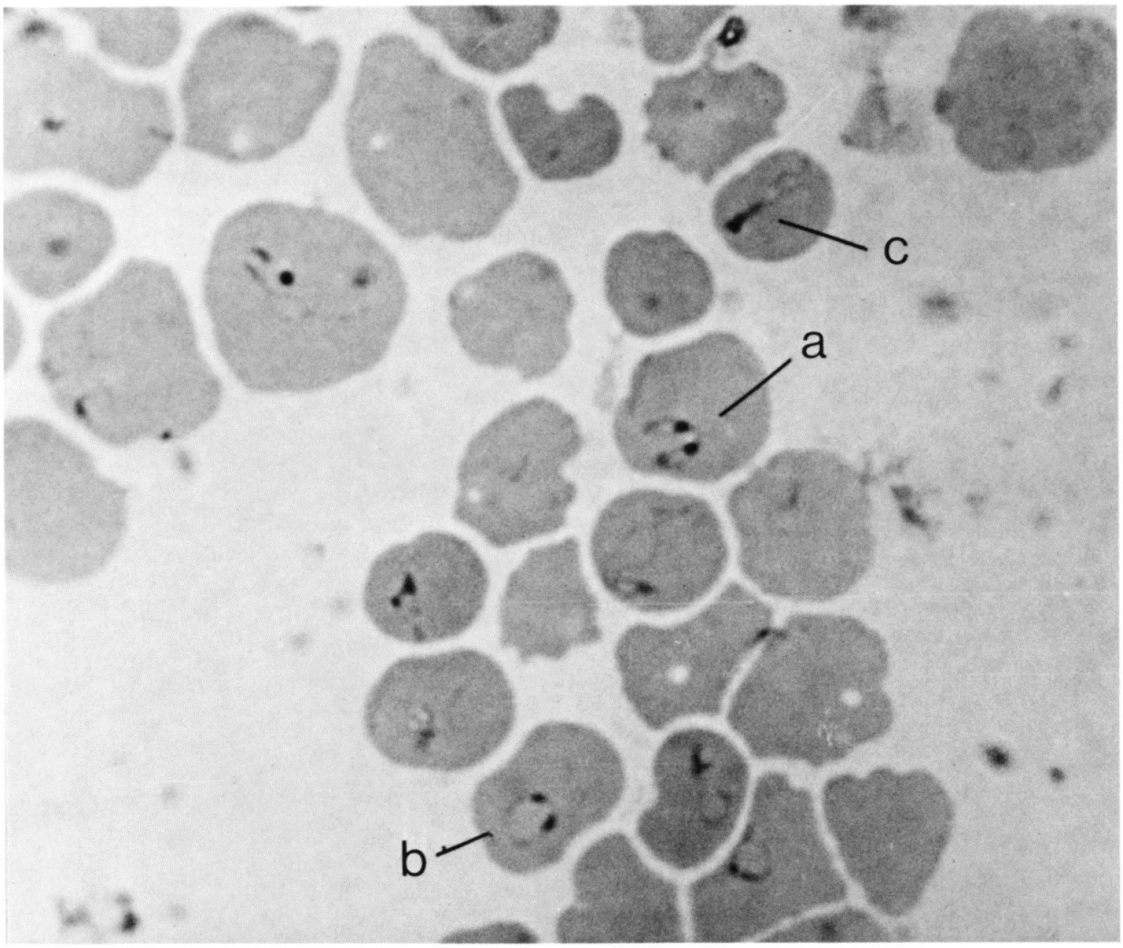


FIGURE 1.7. Blood film from the original description of *B. lotori* from raccoons in Connecticut, USA. a) paired pyriform stages, b) ring stage, and c) amoeboid form. These parasites were not available for genetic characterization so could represent either *B. lotori* or the *B microti*-like sp. (From Anderson et al., 1981).

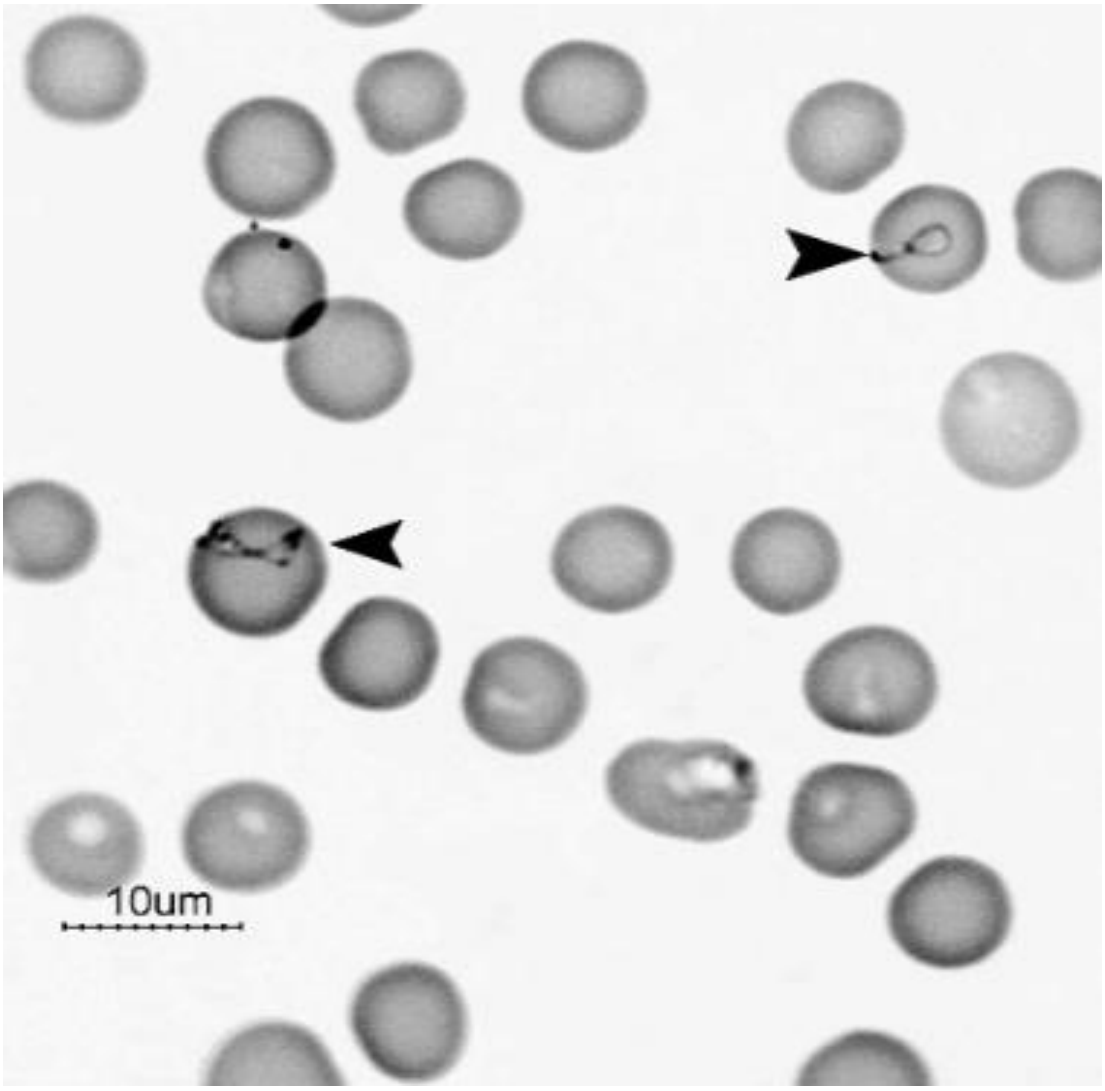


FIGURE 1.8. Blood smear from raccoon from Illinois that had a *Babesia* sp. that was genetically characterized and is now considered to be the type sequence for *B. lotori*. (From Birkenheuer et al., 2006)

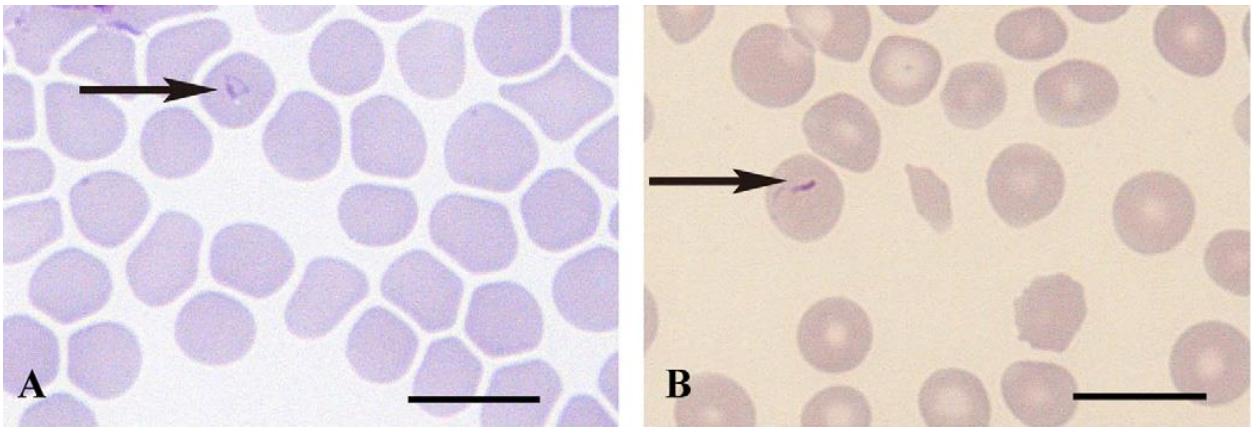


FIGURE 1.9. *Babesia* from raccoon MA230 from Japan. Genetically this parasite was most similar to *B. lotori* shown in Figure 6. (From Jinnai et al., 2009).

Genetic characterization

Molecular identification of raccoon *Babesia* spp. has relied primarily on 18S rRNA gene sequences. Unfortunately, most PCR protocols for this gene will amplify both species and because co-infection was common in a study in North Carolina, amplification and subsequent sequence analysis can be difficult. However, Birkenheuer et al. (2007) developed two different PCR assays that primarily detect the *B. microti*-like sp. or the *Babesia* sensu stricto group, which has been assumed to be only *B. lotori* in raccoons. Although this PCR assay is useful for detecting infections with these two groups of parasites, the amplified region is very short (~230-340 bp) so phylogenetic studies are not possible with these sequences. Because *B. microti* is a human pathogen, there are a few published protocols that are believed to only detect the *B. microti*-like clade, but screening of animals with just these protocols would miss *Babesia* sensu stricto infections.

Prevalence and distribution of Babesia *infections in raccoons from the US*

Babesia spp. infections in raccoons have been noted in multiple locations throughout the Eastern and Midwestern United States (Table 1) (Frerichs and Holbrook, 1970; Shaffer et al., 1978; Anderson et al., 1981; Telford and Forrester, 1991; Goethert and Telford, 2003; Birkenheuer et al., 2006; Birkenheuer et al., 2007). The most notable study was by Birkenheuer et al. (2007) in North Carolina who tested 41 raccoons for *Babesia* with the two PCR assays discussed above. They found high prevalence of infections occurring in raccoons, with 83% infected with *B. microti*-like sp., 90% infected with *B. lotori*, and 76% co-infected with both species. This was one of the few studies that has evaluated co-infection in carnivores infected with *Babesia* species, though some work has been conducted on co-infections in ungulates (Penzhorn, 2006; Birkenheuer et al., 2007). Little work has been done to document the prevalence and distribution of this parasite in US raccoons; however, it is suspected that these piroplasms likely infect raccoons across their native range.

TABLE 1.1. Distribution of genetically confirmed reports of *B. lotori* and *B. microti*-like sp. as well as reports of unidentified *Babesia* sp. in raccoons in North America

Parasite species	**State**	**No pos/No. sampled (%)**	**Testing method**	**Reference**
Babesia lotori	Illinois	1/1 (100)	PCR	Birkenheuer et al., 2006
	North Carolina*	37/41	PCR	Birkenheuer et al., 2006
Babesia microti-like sp.	Massachusetts	1/1	PCR	Goethert and Telford, 2003
	North Carolina	34/41	PCR	Birkenheuer et al., 2007
	Florida	14/17	PCR	Clark et al., 2012
Babesia sp.	Maryland	29/30	Blood stain	Frerichs and Holbrook, 1970
	Tennessee	3/6	Blood stain	Shaffer et al., 1978
	West Virginia	3/10	Blood stain	Shaffer et al., 1978
	Florida	11/33	Blood stain	Shaffer et al., 1978
	Georgia	2/10	Blood stain	Shaffer et al., 1978
	Virginia	4/10	Blood stain	Shaffer et al., 1978
	Texas	15/25	Blood stain	Shaffer et al., 1978
	Connecticut	12/14	Blood stain	Anderson et al., 1981
	Florida	82/184	Blood stain	Telford and Forrester, 1991
	Malibu Canyon, Santa Monica Mtns., Los Angeles , California	1 of unknown	Blood stain	Wood 1952

*The PCR assay used will amplify all *Babesia* sensu stricto; however, sequences from 7 samples matched *B. lotori*.

Prevalence and distribution of Babesia *infections in raccoons outside of the US*

Raccoons have been introduced to numerous European and Asian countries through a diversity of ways including: escaped/released pets, escaped/released from fur farms, and intentional release for hunting (Beltran-Beck et al., 2012). Densities in Europe are generally lower than those in the United States; however, in some places, such as Germany, the densities have gotten high enough that raccoons are considered a nuisance species (Beltrán-Beck et al., 2012; Fischer et al., 2015). *Babesia* infections have not been documented in European raccoons but this is likely due to a lack of surveillance. A related parasite, a *Babesia microti*-like species, has also been noted in European dogs (*Canis lupus familiaris*) and red foxes (*Vulpes vulpes*) (Baneth et al., 2015). This parasite was initially named *Theileria annae* due to the parasite not resembling any species from the *Babesia* sensu stricto group; however, it was recently renamed *Babesia vulpes* and is closely related to the *B. microti*-like clade of carnivores (Baneth et al., 2015).

In Japan, raccoons are an invasive species that were introduced in the 1970's after a popular television show "Rascal Raccoon" prompted demands for raccoon as pets (Ikeda et al., 2004). At least three *Babesia* spp. have been documented in Japanese raccoons, a *Babesia microti*-like sp. similar to the one found in the US, a species closely related to *B. lotori*, and a novel *Babesia* species (Kawabuchi et al., 2005; Jinnai et al., 2009). The novel strains have only been documented in Japan (Jinnai et al., 2009). Although these studies confirm that raccoons in Japan harbor *Babesia* infections, the prevalence is much lower (3-10%) than in raccoons from the US (Kawabuchi et al., 2005; Jinnai et al., 2009). It is suspected that *Babesia* infections in raccoons are transmitted by ticks, however Japan hosts a different suite of ticks. It is possible that

some of these tick species are related enough to transmit these *Babesia* spp. Other methods of transmission such as fighting between conspecifics, or vertical transmission may also maintain infections in Japanese raccoons (Jinnai et al., 2009). These alternative transmission routes may be the reason prevalence is much lower in raccoons from Japan compared to the United States. It is also possible that the novel *Babesia* sp. detected is a Japanese native and is now utilizing the raccoon as a new host.

Clinical signs/impacts of Babesia *infections in raccoons*

To date there has only been one possible case of clinical disease in a raccoon infected with *Babesia*. In Illinois, a raccoon that was nonambulatory and experiencing pronounced anemia, hypoproteinemia, hypalbuminemia, and elevated alanine aminotransferase was infected with *B. lotori* (Birkenheuer et al., 2006). The raccoon was treated and released; however, it is unknown if the clinical signs were due to *Babesia* (although parasitemias were low) or a possible undiagnosed illness.

Splenomegaly is a common symptom associated with infection with *Babesia* species in many hosts (Mierzejewska et al., 2014; Solano-Gallego et al., 2016). Because of this, Kawabuchi et al. (2005) tested raccoons with splenomegaly for *Babesia* species infections and found that 8% were positive *for Babesia* spp.; however, no raccoons without splenomegaly were tested.

No further studies or experimental trials have been conducted on the impacts of *Babesia* infections on raccoons; however, it is suspected that disease is rare and raccoons likely develop chronic infections once infected.

Ticks associated with raccoons

Babesia spp. are all assumed to utilize tick vectors, specifically ixodid ticks, for transmission, even if they can also use alternative transmission routes (Uilenberg, 2006; Lack et al., 2012; Schnittger et al., 2012; Yabsley and Shock, 2013). In the United States, there have been numerous studies to characterize the tick fauna present on raccoons and in most studies the prevalence and diversity is high. The most common tick species reported include: *Ixodes texanus, I. cookei, I. scapularis, I. affinis, Dermacentor variabilis, Amblyomma americanum,* and *A. maculatum;* however, many of these tick species have ranges that do not all overlap and different seasonal phenologies that may result in only certain ticks found on raccoons during a particular season (Dennis et al., 1994; Pung et al., 1994; Ouellette et al., 1997; Yabsley et al., 2008). *Ixodes texanus* is of particular interest as it is one of the most commonly detected tick species on raccoons, has been found on young raccoons restricted to nests, and has expansive geographic range with reports from the eastern US to the western coast including Alaska and California (Pung et al., 1994; Durden et al., 1996; Ouellette et al., 1997; Gabriel et al., 2009; Durden et al., 2016).

Seasonality of tick infestation on raccoons varies based on the tick species and stage. For example, in Georgia adult *I. texanus* and *A. americanum* are generally reported on raccoons in the summer (May-September), *I. scapularis* adults in the winter, and *Dermacentor variabilis* in the spring and summer (Anderson and Magnarelli, 1981; Pung et al., 1994). Because the two *Babesia* species in raccoons are found in high prevalence, at least in North Carolina, the vector is presumed to be a common tick of raccoons.

Currently no routes of transmission have been identified for raccoon *Babesia* spp., but it is presumed to be an *Ixodes* spp. In a study on vector competence of *I. scapularis* for *B. microti*, several nymphs that had fed on raccoons and opossums as larva were infected with the raccoon *B. microti*-like sp. (Hersh et al., 2012). These nymphal ticks could have acquired the infections while feeding on the raccoons or opossums, or were transovarially infected. Also, one study found that two of four young raccoons (no age given) confined to a chimney were positive for *Babesia* and *Ixodes texanus* ticks were found on two them. The mother was also infected, but it is unknown if the infection was vertically transmitted to the young raccoons or transmitted by the ticks (Anderson et al., 1981).

Transmission routes

For *Babesia* spp. which have known life cycles, all are transmitted by ticks (mostly ixodid ticks); however, alternative methods of transmission such as vertical, through biting or fighting, or through blood transfusions have been documented (Homer et al., 2000; Uilenberg, 2006; Hunfeld et al., 2008; Yeagley et al., 2009).

Other transmission routes have been documented for other *Babesia* spp. and piroplasms. Vertical transmission (transmission from mother to young) has been documented for many species of *Babesia*. *Babesia microti* have been vertically transmitted in experimentally infected lab rodents, shrews, and one human case in an infant (Bednarska et al., 2015; Tolkacz et al., 2017). Cases of vertical transmission in the sensu stricto *Babesia* clade include a case of *Babesia gibsoni* in beagle puppies, *Babesia canis canis* in shepherd puppies, and *Babesia bovis* in a cattle calf (Yeruham et al., 2003; Fukumoto et al., 2005; Joseph et al., 2012; Mierzejewska et al., 2014; Brown et al., 2015; Adaszek et al., 2016; Costa et al., 2016). For raccoons, most studies on

Babesia have been conducted on adult raccoons, with little known about infection of neonate and young raccoons. The only exception being the Anderson et al. (1981) study that found infections in a few young, nest-confined, raccoons.

Biting and fighting that leads to blood transfer is another possible route of transmission. Evidence indicates that *Babesia gibsoni*, a species of *Babesia* found in fighting dogs, is transmitted via wounds in the oral cavity, allowing for transmission of infected blood between individuals during fighting (Yeagley et al., 2009). It is suggested that raccoon *Babesia* species may be capable of utilizing this transmission strategy, which is of particular interest because of an incident of the death of a captive maned wolf (*Chrysocyon brachyurus*) from babesiosis following a fight with a raccoon (Birkenheuer, unpublished data). The maned wolf was in a Missouri zoo and died due to babesiosis caused by a *Babesia* species closely related to *Babesia lotori* (Genbank accession no. KR017880, *Babesia* sp. maned wolf) (Birkenheuer, unpublished data). Because of the genetic similarity of the parasite with *B. lotori*, there is concern that the fight led to transmission and that *B. lotori* from raccoons can be pathogenic to certain species, such as maned wolves. *Babesia* infections have been previously reported in maned wolves but *Babesia canis* was the species diagnosed (Cansi et al., 2012; Phair et al., 2012). Maned wolves infected with *Babesia* sp. experience severe disease and may have lethargy, vomiting, slight head tilt, possible loss of vision, dark red urine, severe anemia, and eventually more severe signs such as respiratory, digestive, and neurologic system failures which eventually lead to death (Cansi et al., 2012; Phair et al., 2012). As this is a near threatened species, knowledge on *Babesia* transmission to maned wolves is needed and additional data on the distribution of *B. lotori* in raccoons may assist with prevention of infection in captive maned wolves (IUCN 2010—International Union of Nature and Natural Resources).

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CHAPTER 2

PREVALENCE, DIVERSITY, AND DISTRIBTUION OF PIROPLASMS IN RACCOONS (*PROCYON LOTOR*) FROM SELECTED AREAS OF THE UNITED STATES AND CANADA

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ABSTRACT

Babesia species are intraerythrocitic protozoan parasites that can infect a variety of hosts, including raccoons (*Procyon lotor*). Two species of *Babesia* have been found to infect raccoons, including *Babesia lotori* (*Babesia* sensu stricto clade) and *Babesia microti*-like sp. (*B. microti*-like in carnivores). The goal of this study was to provide more knowledge on the diversity, distribution, and prevalence of *Babesia* piroplasms in raccoons. We tested raccoons from selected regions in the United States and Canada for the presence of *Babesia* sensu stricto and *Babesia microti*-like sp. piroplasms. Infections of *Babesia microti*-like sp. were found in all locations, while *Babesia* sensu stricto infections were more common in the Southeastern United States (20-45% [145/519]). Co-infections were common. Further sequencing of the partial 18S rRNA and cytochrome oxidase subunit 1 (*cox1*) genes was conducted on samples to evaluate intraspecific variation of each species. Sequencing analyses revealed the discovery of two new *Babesia* species: a novel *Babesia* sensu stricto sp. in the US, and a novel western *Babesia* species in raccoons. Further analyses will be needed to identify these novel species; however, this study indicates there are at least four species of piroplasms infecting raccoons, possibly five: a *Babesia microti*-like sp., *Babesia lotori*, a novel *Babesia* sensu stricto sp., a novel western *Babesia* sp., and the *Babesia* sensu stricto species in Japan.

INTRODUCTION

The order Piroplasmida is a diverse group of intracellular parasites, many of which can cause significant disease in humans, domestic animals, and wildlife. Historically these parasites were identified based on their host or morphologic characteristics (i.e. shape or size), but recent molecular studies have revealed a much greater host-range and diversity of these parasites than

previously recognized (Gray et al., 2002; Criado-Fornelio et al., 2003; Allsopp and Allsopp, 2006; Hunfeld et al., 2008; Lack et al., 2012; Schnitteger et al., 2012). The Piroplasmida group is currently polyphyletic and includes a *Cytauxzoon* spp. group, a group of most *Theileria* spp., a separate lineage with just *Theileria equi,* and the genus *Babesia* divided into three distinct clades (*Babesia* sensu stricto, Western *Babesia* group, and *Babesia microti/*related *Babesia* group), (Criado-Fornelio et al., 2003; Allsopp and Allsopp, 2006; Lack et al., 2012; Schnittger et al., 2012; Shreeg et al., 2016).

Many wildlife species in North America, such as the Northern raccoon (*Procyon lotor*), Florida puma (*Puma concolor coryi*), and river otter (*Lontra canadensis*), have high prevalence of *Babesia* spp., but there is relatively little known about the natural history, geographic distribution, and diversity of *Babesia* in these hosts (Birkenheuer et al., 2006; Yabsley et al., 2006; Birkenheuer et al., 2007a; Birkenheuer et al., 2007b). Raccoons are of particular interest because they have a large natural geographic range within North America, have established introduced populations in numerous European and Asian countries, and are known hosts for at least two *Babesia* species: *B. lotori* (in the *Babesia* sensu stricto group) and a *Babesia* sp. closely related to *B. microti* (in *Babesia microti* group) and one study noted that co-infections were common (Frerichs and Holbrook, 1970; Anderson et al., 1981; Goethert and Telford, 2003; Kawabuchi et al., 2005; Birkenheuer et al., 2007a; Jinnai et al., 2009; Beltran-Beck et al., 2012). Although several studies have reported *Babesia* infections in raccoons, most of these studies relied on morphologic identification of the parasite, which lacks sensitivity and the specificity needed to discern the two morphologically similar *Babesia* spp. (Frerichs and Holbrook, 1970; Anderson et al., 1981; Goethert and Telford, 2003; Birkenheuer et al., 2006; Birkenheuer et al., 2007a).

The nomenclature of these raccoon *Babesia* piroplasms is also historically complicated. Piroplasms initially detected in raccoons were reported as *Babesia procyoni* or *Babesia procyonis*; however, a large *Babesia* spp. (>3 µm) of Eurasian raccoon dogs (*Nyctereutes procyonoides*) was also named *Babesia procyoni* (Frerichs and Holbrook, 1970; Anderson et al., 1981; Birkenheuer et al., 2006). To address this issue, Anderson et al. (1981) described the small parasite of raccoons as *B. lotori*. Subsequent molecular studies detected a *Babesia microti*-like species, which currently has no official nomenclature and is reported as *B. microti*-like sp., *B. microti*, *B.* cf *microti*, or a *Babesia* sp. in the *"microti*-like in carnivores" clade (Goethert and Telford, 2003; Birkenheuer et al., 2007a). In Japan, where raccoons have become established, molecular testing has revealed a third novel *Babesia* sp. that is also in the *Babesia* sensu stricto group, as well as presence of the *B. microti*-like sp. and a *Babesia* species similar to *B. lotori* (Jinnai et al., 2009).

For *Babesia* spp. with known life cycles, ticks, primarily ixodid ticks, are the vectors; however, other methods of transmission can include vertically from mother to young, biting and fighting, and blood transfusions (Uilenberg, 2006; Hunfeld et al., 2008; Yeagley et al., 2009; Lack et al., 2012; Schnittger et al., 2012; Binda, 2013; Yabsley and Shock, 2013; Bednarska et al., 2015). Currently there is no known tick vector for any of the *Babesia* spp. from raccoons in the United States and Japan. The recent death of a captive maned wolf (*Chrysocyon brachyurus*) from a *Babesia* most closely related to *B. lotori* has caused speculation that this raccoon piroplasm may also be transmitted through biting, as the maned wolf was seen fighting with a wild raccoon (Birkenheuer unpublished).

The objectives of this study were to investigate the prevalence, distribution, and diversity of *Babesia* spp. in raccoons at selected sites in the United States and Canada. We also amplified and sequenced partial 18S rRNA and cytochrome oxidase subunit I (*cox1*) genes to evaluate intra-specific variation of the *Babesia* spp. in raccoons from different geographic regions.

METHODS

Sample Collection

Samples were opportunistically obtained from various locations in the United States (Georgia, Florida, West Virginia, Pennsylvania, Minnesota, Missouri, Colorado, Texas, and California) and Canada (Ontario and Nova Scotia). Samples were obtained from various collaborators (rehabilitation facilities, vet clinics, etc.) from June 2015-January 2017. The sample type varied according to availability, but most samples were either whole blood or spleen. Other organs such as liver or lung were used if these samples were unavailable. Samples were stored in -20 C freezer until DNA extraction. No animals were euthanized for the purposes of this study but 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 or 200 µl of whole blood using a commercial kit per manufacturer's instructions (DNEasy Blood and Tissue kit, Qiagen, Hilden, Germany). Two different PCR (polymerase chain reaction) assays were utilized to target the V4 region of the 18S rRNA gene of *Babesia* as described in Birkenheuer et al. (2003, 2007a). The primers used to amplify *Babesia microti*-like spp. were Bmlike F and 793-772 R, which were expected to produce a 229 base pair amplicon. Samples were amplified in a BioRad DNA Engine

Peltier Thermal Cycler (Bio-Rad Laboratories Incorporated, Foster City, CA) using the following cycle parameters: 94 C for 5 minutes followed by 49 cycles of 94 C for 45 seconds, 56 C for 45 seconds, and 72 C for 45 seconds, with a final extension of 72 for 5 minutes. To amplify *Babesia* sensu stricto spp., primers 455-479 F and 793-772 R were used and the expected amplicon was 341 base pairs. Cycling parameters were 94 C for 3 minutes followed by 44 cycles of 94 C for 30 seconds, 60 C for 30 seconds, 72 C for 30 seconds, with a final extension of 72 C for 5 minutes. The size of this amplicon was carefully examined because it was expected that, due to sequence similarity within the primer-binding region, the *B. microti*-like species as well as the western *Babesia* group may be amplified if present in high concentration, but the amplicon size should be 371bp instead of 341bp (Birkenheuer et al., 2003, 2007). Primers sequences are listed in Table 2.1.

Standard precautions were taken to prevent or detect contamination in all steps, such as performance of DNA extraction, PCR reaction setup, and product analysis being conducted in distinct and designated laboratory areas. Negative water controls were included in each set of DNA extractions and for each set of PCR reactions. Positive controls were created from a pooled blood sample which contained both sequence confirmed *B. lotori* and the *B. microti*-like sp. Amplicons were electrophoresed in a GelRed stained 1.5% agarose gel.

In order to determine phylogenetic relationships of selected samples, amplification and sequencing of near full length 18S rRNA gene were conducted using primers 5-22 F and 1661 R (1,650 bp amplicon) (Table 2.1) (Birkenheuer et al., 2007a). Cycling parameters were 95 C for 5 minutes followed by 50 cycles of 95 C for 20 seconds, 55 C for 30 seconds, 72 C for 2 minutes, and a final extension step of 72 for 5 minutes. To investigate if any intraspecific variation occurred between the *Babesia* spp. in different geographic regions, amplification of the

cytochrome c oxidase subunit 1 (*cox1*) gene was conducted for a subset of samples. Primers Babcox1F and Babcox1R which amplify a 1,085 bp amplicon were used (Table 2.1) (Schreeg et al., 2016). Cycling parameters were 95 C for 5 minutes followed by 45 cycles of 95 C for 20 seconds, 50 C for 30 seconds, 68 C for 1 minute and 30 seconds, and a final extension of 72 C for 5 minutes.

Because of the lack of *cox1* sequences from representatives in the western *Babesia* group and relatives, we obtained samples of *Babesia* sp. from a Fallow deer (*Dama dama*) and *Theileria youngi* from a dusky-footed woodrat (*Neotoma fuscipes*), and *Babesia* spp. from three spotted hyenas (*Crocuta crocuta*) and a lion (*Panthera leo*) from Zambia (Williams et al., 2014; Kauffmann et al., 2017).

Sequencing and Phylogenetic Analysis

Selected amplicons were purified from gels using a Qiagen gel-purification kit (QIAquick gel extraction kit, Hilden Germany), and bi-directionally sequenced at the University of Georgia Genomics Facility (Athens, GA). Chromatograms were analyzed using Geneious (Biomatters Limited, Auckland, New Zealand). Sequences were aligned in MEGA (Molecular Evolutionary Genetics Analysis), visually analyzed, and cropped to include as many sequences as possible. Phylogenetic trees were constructed using the neighbor-joining algorithm in MEGA. *Plasmodium falciparum* was used as an outgroup, similar to that of other phylogenetic studies on Piroplasmida (Criado-Fornelio et al., 2003; Shreeg et al., 2016).

Sequences chosen from Genbank were based on previous studies of *Babesia* infections in raccoons and other carnivores (Kjemtrup et al., 2000; Kjemtrup et al., 2001; Kjemtrup et al., 2006; Yabsley et al., 2006; Jinnai et al., 2009; Baneth et al., 2015; Shreeg et al., 2016). Percent identities for both the 18S and *cox1* dataset were calculated using the distance estimation analysis in MEGA using the Tamura 3-paratemter model and pairwise deletion.

Statistical Analyses

A Fisher's exact test was used to compare the prevalence of *B. microti*-like sp. and *Babesia* sensu-stricto infections between locations. Prevalence for *B. microti*-like sp., *Babesia lotori*, and coinfection were determined at each location, as well as an overall prevalence, and 95% confidence intervals were calculated.

RESULTS

Prevalence and distribution of the two Babesia *groups*

Based on the initial screening PCR targeting the 18S rRNA gene, the prevalence of *Babesia* infections in raccoons at all sites was 76% [395/519] (95% confidence interval: 69.8-77.4) (Table 2.2). Overall, 73.6% [383/519] of raccoons were positive for *B. microti*-like sp. infections while 27.5% [143/519] were positive with the *Babesia* sensu stricto species PCR. Only 24.9% [129/519] of all tested raccoons were co-infected (positive with both PCR assays), with the maximum coinfection rate being 43.8%.

There were significant differences in prevalence among sample locations (Table 2.2, Figure 2.1). Generally, the prevalence of the *Babesia* sensu stricto group was highest in the southeastern and Mid-Atlantic United States (20-40%) compared to the midwestern United States and Ontario, Canada (4-20%), and the western United States (0-30%) (Figure 2.1, Table 2.3). *Babesia microti*-like sp. infections were common across all study sites (40-100% prevalence), except in Nova Scotia where only 6% [5/80] raccoons were positive.

Sequence Analysis

A total of 43 samples were sequenced and we identified four groups of *Babesia* spp. including *B. lotori, B. microti*-like sp., a novel *B.* sensu stricto sp., and a novel western *Babesia* sp. Screening PCR results were not always concurrent with sequencing results as it seems the novel *Babesia* sensu stricto sp. and the novel western *Babesia* sp. are both amplified by the *B.* sensu stricto screening PCR, and if co-infections occurred, some samples amplified one species vs. another species based on the gene targeted (Table 2.4).

18S rRNA gene

Phylogenetic analysis of 17 samples that had near-full length sequence (minimum of 1655 bp) revealed four distinct groups. Seven samples grouped with numerous sequences of the *B. microti*-like sp. in raccoons from the USA and Japan. This group was distinct from *B. microti*-like species in other carnivores (e.g., raccoon dog (*Nyctereutes procyonoides*), fox (*Vulpes vulpes*), river otter (*Lontra Canadensis*), badger (*Taxidea taxus*), as well as *Babesia microti* sequences from humans and rodents. Intraspecific variation among the *B. microti*-like sp. from raccoons, regardless of origin, was low (98.8-100% identity) (Appendix 1). Similarity of this raccoon *B. microti*-like sp. compared to the *B. microti*-like sp. from other carnivores was much lower (93-98% identity) (Appendix 1). One of our sequences was a member of the western

Babesia group and was most similar to *B. conradae* and *Babesia duncani* (96-97%). The remaining six sequences were similar to *B. lotori* but were phylogenetically separated into two clades, but bootstrap support for these two clades was low (48%) making it difficult to say for surety whether or not this represents two species or one. One group contained our sequences from West Virginia (USA), Colorado (USA), and Ontario (Canada) and five previously published sequences from Japan. The other group contained *B. lotori* from a raccoon from Illinois (USA) (DQ028958, Birkenheuer et al., 2006), a *Babesia* sp. from a captive maned wolf (KR017880), and raccoons in our study from Pennsylvania, Missouri, and California (USA). Sequences from the Japanese/North American group and the *Babesia lotori* group were 99% similar to one another. A previously noted novel *Babesia* sensu stricto species reported in Japanese raccoons (Jinnai et al., 2009) was also noted in our phylogenetic analysis, but none of our sequences were contained within that group (Figure 2.2). This Japanese-only group was only 96-97% similar to the other raccoon *Babesia* sensu stricto sequences.

Cox1 gene

We obtained *cox1* sequences (minimum of 1080 bp) from 40 samples. Phylogenetic analysis resulted in four distinct groups of *Babesia* infecting raccoons and the phylogenetic relationships were similar to our 18S rRNA gene analysis (Figure 2.3). However, unfortunately, there are no *cox1* sequences for any of the *Babesia* spp. previously reported from Japan. Similar to the 18S rRNA gene analysis, the *B. microti*-like sp. group was a well supported clade (100%) that grouped separately from *B. vulpes* in foxes and *B. microti* from humans. Although there was more intraspecific variation among the *B. microti*-like sp. (97-100% identity) compared to our 18S rRNA analysis, there was no association with geographic origin. *Babesia vulpes,* the most similar species to the raccoon *Babesia microti*-like sp., differed from the raccoon sequences by at

least 10%. Five of our sequences were included in the western *Babesia* clade. The sequences were most similar to a *Babesia* in spotted hyenas from Zambia (RACH6, Rachhym and Waterhym) (80-81% identity) followed by *Babesia* in a Fallow deer (79-80% identity). Phylogenetically, these raccoon sequences were in a sister clade to the *Babesia* sp. from a Fallow deer, although bootstrap support for several clades within this group were low (<80%). Interestingly, there was strong support (100%) for geographic variation within the raccoon Western *Babesia* group with the two Georgia raccoon samples occurring in a sister group to the three California raccoon samples. The intraspecific percent similarity within the two geographic regions was high (99-100% identity), whereas the interspecific similarity between the two geographic regions was only 90.1-90.4%. The remaining sequences formed two clades, similar to the 18S rRNA results, although the bootstrap support was low (46%). Within the *B. lotori* group there was high support (100%) for geographic variation with eastern samples from Pennsylvania, West Virginia, and Illinois being a sister group to sequences from Missouri, Idaho, and California. However, the percent identity between these two groups was high (97-99%). The other *Babesia* sensu stricto sp. group had high variability (91-100% identity), but this was also due to the apparent presence of geographic variation with one sample from Colorado being separated from the other *Babesia* sequences from Pennsylvania and Minnesota (USA) and Ontario (Canada). The Colorado sequence only had 91% identity to other samples within this group.

DISCUSSION

We found that raccoons are commonly infected with *Babesia* species in many parts of the USA and Canada and, in some areas, coinfections are common. Previously, *Babesia* infections had been reported in raccoons from multiple locations in the United States; however, few of these studies have used molecular assays to distinguish *Babesia* species (Anderson et al., 1981,Telford and Forrester, 1991; Birkenheuer et al., 2006; Birkenheuer et al., 2007a). This is especially important because the two previously detected *Babesia* spp. in raccoons (*Babesia lotori* and *Babesia microti*-like sp.) are morphologically similar so molecular analysis is required to identify the species present.

We found that the most common and widespread *Babesia* sp. in raccoons was the *Babesia microti*-like sp. Previously this species had been reported from a single raccoon in Massachusetts and in a high prevalence in two surveys in North Carolina and Florida (Birkenheuer et al., 2007a, Clark et al., 2012). Similarly, we found a high prevalence of *Babesia microti*-like sp. in some regions, especially in the southeastern United States (67-100%), although we confirmed infections in a number of locations in midwestern and western states.

The *Babesia microti* group is a diverse group that includes parasites of rodents and humans as well as numerous carnivore species. There is mounting evidence that these parasites are separate species and that some have a high degree of host-specificity (Criado-Fornelio et al., 2003; Allsopp and Allsopp, 2006; Lack et al., 2012; Schnittger et al., 2012; Yabsley and Shock, 2013; Shreeg et al., 2016). Our 18S rRNA and *cox1* gene data indicate that the parasites infecting raccoons are distinct from the other carnivore-infecting *B. microti*-like species. Also, the intraspecific variability of the raccoon *B. microti*-like sp. was low with 98-100% sequence identity for the 18S rRNA gene and 97-100% sequence identity for the *cox1* gene. This group

was at least 10% different at these loci from the closest available *cox1* sequence from *B. vulpes* in foxes and dogs in Europe (Baneth et al., 2015). Based on 18S rRNA gene analysis we found no separation among the sequence from raccoons in the US or Japan, but analysis of additional gene targets would be more ideal for looking at variation within the group, including the *cox1* gene which was unavailable for Japanese samples (Jinnai et al., 2009). There are limited sequences of the gene from 3 raccoons in Florida (USA) and Japan (n=1). Japanese researchers have sequenced the chaperonin-containing t-complex polypeptide l (CCTη) gene for *several B. microti*-like samples, including one sample from a Japanese raccoon, but this gene target has not been sequenced from US or Canadian samples (Nakajima et al., 2009).

In an effort to clarify this group of *B. microti*-like species, Baneth et al. (2015) recently proposed the name *B. vulpes* for a *Babesia* sp. of dogs and foxes that had previously been published under numerous names including *Babesia* Spanish dog isolate, *Babesia microti*-like, *Babesia annae*, *Theileria annae*, and *Babesia* cf. *microti*. Although we believe there is sufficient evidence for the raccoon *B. microti*-like species to be a unique species that appears to be specific to raccoons, we have chosen not to propose a new name until a holotype can be obtained and deposited as required by the International Code of Zoological Nomenclature (Harris, 2016). In addition, in keeping with tradition of this group, the new species would be in the genus *Babesia*; however, numerous analyses, including our own, indicate that this *B. microti* group, as well as other related small babesids, should be reclassified into a new genus which should be completed in the future (Criado-Fornelio et al., 2003; Allsopp and Allsopp, 2006; Lack et al., 2012; Schnittger et al., 2012; Yabsley and Shock, 2013; Shreeg et al., 2016).

Our initial screen results based on 18S screening PCR found that the *Babesia* sensu stricto group was most common in raccoons in the southestern US, but infections were noted at all sampled areas except for Texas, which had a low sample size (n=3), and Nova Scotia (Canada). We had assumed that these positives were *B. lotori*, the only other *Babesia* sp. reported from raccoons in the US, or the novel *Babesia* species reported from raccoons in Japan (although it is unknown if the *Babesia* in Japanese raccoons was from the US or was acquired by raccoons after their introduction to Japan) (Anderson et al., 1981; Birkenheuer et al., 2006; Birkenheuer et al., 2007a; Jinnai et al., 2009; Clark et al., 2012). However, sequence analysis of selected samples from our study showed that there were three species of *Babesia* being amplified by the *B.* sensu stricto screening PCR. These data highlight the issue with using genus- or group-wide molecular assays without subsequent sequence analysis to confirm or identify the species detected.

One of the *Babesia* sensu stricto group parasites we detected was *B. lotori*, previously only noted to occur in raccoons in Illinois and North Carolina. However, recently a genetically similar parsite was also reported in a sick captive maned wolf (*Chrysocyon brachyurus*) from Missouri, possibly expanding the range (KR017880, Birkenheurer, unpublished data) (Birkenheuer et al., 2006; Birkenheuer et al., 2007a). Interestingly, based on *cox1* phylogenetic analysis, there was evidence of geographic differences between eastern and western locations among the *B. lotori*-like sp. sequences. One group included the original *B. lotori* sequence from Illinois as well as sequences in our study from Pennsylvania and West Virginia, while the other group included the *Babesia* sp. from the maned wolf, as well as sequences from the western and central United States, including Missouri, Idaho, and California. The eastern and western groups were related (96.8-99% similar) but the within-group sequence similarity was much higher (98.8-99.8%). The mechanisms of this geographic variation are unknown but could be related to

different tick vectors among these regions (although there are some tick vectors such as *I. texanus* that occur throughout North America), or through variation after raccoon populations became isolated (Dennis et al., 1994; Pung et al., 1994; Ouellette et al., 1997; Yabsley et al., 2008). Currently there are numerous subspecies of raccoons recognized in North America (e.g., eastern raccoons are *Procyon lotor lotor* while western raccoons, such as Missouri or California, are Upper Mississippi Valley raccoons (*Procyon lotor hirtus*), California raccoons (*Procyon lotor psora*) or short-face raccoons *(Procyon lotor simus*)). However, more research is needed to determine how these subspecies could affect *Babesia* infections (Lotze et al. 1979). Additional data are needed to better understand the diversity within the *B. lotori*-group but at this time the eastern and western genetic variants appear to be a single species (*B. lotori*). Genetic variants of *B. lotori* are widespread in the US and appear to not be specific to only raccoons. Thus, some species, such as the maned wolf, may be a risk if they become infected.

Within the *Babesia* sensu stricto group, there seems to be a novel species that is closely related to *B. lotori*. The 18S rRNA analysis showed that some samples from West Virginia, Colorado, and Ontario (Canada) were closly related to several sequences from Japanense raccoons (Jinnai et al., 2009). Although bootstrap support for seperation of this group from *B. lotori* was low for both gene analyses (46%), the groups were only 81-83% similar based on *cox1* sequences suggesting that they are distinct, depsite the phylogenetic relationship being unresolved. Unfourtunatly, for the *cox1* gene analysis, we were unable to include the Japanese samples as sequencing of this gene has not been conducted. However, based on our 18S rRNA data, this group of *Babesia* species previously reported in Japan likely was introduced with the introduction of the raccoon.

Data from Jinnai et al., (2009) also indicated that there was a novel clade of *Babesia* in raccoons in Japan. In our 18S analysis, this group remained distinct and none of our US or Canadian samples occurred in this group. Thus, the orign of this group of *Babesia* is currently unknown. It could be that this species is endemic to North America and we failed to detect it due to low samples sizes in each state, or we simply did not sample where this parasite was present; however, the four groups of *Babesia* we did detect were found in the eastern and western US, suggesting that these parasites are not geographically isolated or restricted. It is also possible that this novel *Babesia* group is native to Japan and has now begun to infect raccoons; additional surveillance of possible Babesia hosts in Japan may ultimately discover the natural native host.

The final group of *Babesia* we detected belonged to the group known as the 'western piroplasms' or 'western *Babesia*, named because members of this group were first detected in various host species in California (Kjemtrup et al., 2006). The Western *Babesia* group is a relatively new group that includes species of medical and veterinary importance, but it is poorly understood relative to their natural history (i.e., no known vectors have been identified for any members). Phylogenetically, this group is most similar to *Theileria* and *Cytauxzoon* spp.; however, the members of this group that have been named were named *Babesia*, e.g., *Babesia duncani, Babesia conradae, B. lengau, B. poelea,* etc. (Criado-Fornelio et al., 2003; Kjemtrup et al., 2006;Yabsley et al., 2006; Shreeg et al., 2016). Not only does phylogenetic data on numerous gene targets suggest this group is not a member of the *Babesia* genus, the mitochondrial genome structure of *B. conradae* supports that this group is a novel genus (Shreeg et al., 2016). Our data show that raccoons in Georgia and California are infected with a parasite within this group but their phylogenetic relationships to other species remains unresolved in both 18S rRNA and *cox1* gene analyses. The *cox1* sequence data indicated that there is geographic separation of the

Georgia and California sequences, consistent with the other two *Babesia* sensu stricto groups. There was 9.2-9.6% difference in the *cox1* sequences of these two geographic groups as well as 100% bootstrap support for separation.In recent years numerous new parasites have been identified from this group, but it is hoped that as more sequences are included in future analyses, the relationships within this interesting group will become more apparent (Kjemtrup et al., 2000; Yabsley et al., 2006; Shreeg et al., 2016). Further analysis of multiple gene targets and morphologic features will be needed before a definitive new species can be named within this group.

Currently there is no transmission route known for any of the *Babesia* species in raccoons. The *Babesia microti*-like sp. was widespread and most populations had a high prevalence. This group of parasites is also found in raccoons from Japan, but the prevalence is much lower. All *Babesia* with known life cycles are transmitted by tick vectors, typically ixodid ticks; however, multiple other routes of transmission such as vertical from mother to young, blood transfusions, and transmission during fighting in wounds in the oral cavity have also been noted (Homer et al., 2000; Uilenberg, 2006; Hunfeld et al., 2008; Yeagley et al., 2009). If the *B. microti*-like species is transmitted by ticks, the vector(s) must be widespread or the parasite must use multiple species of ticks. The other three *Babesia* species detected in raccoons from the US and Canada occurred in much lower prevalences but they were all also geographically widespread. Most work on tick communities on raccoons has been conducted in the Eastern US where multiple species have been found including *Ixodes texanus, I. scapularis, I. affinis, Dermacentor variabilis, Amblyoma americanum,* and *A. maculatum*, with *I. texanus* being of considerable interest as it is not only a common nest species of raccoons, but it also has a wide distribution, ranging from the eastern US to the west coast and Alaska (Anderson and

Magnarelli, 1980; Dennis et al., 1994; Pung et al., 1994; Ouellette et al., 1997; Yabsley et al., 2008; Gabriel et al., 2009; Durden et al., 2016; Ondrejicka et al., 2017). Although fewer studies have investigated ticks on raccoons in the western US, several tick species have been reported including *I. texanus, I. rugosus, I. pacificus,* and *D. variablis* (Gregson, 1956; Furman and Loomis, 1984).

In conclusion, our study was the first to evaluate the prevalence and distribution of *Babesia* in raccoons across a large geographic scale. This study has greatly expanded our knowledge of *Babesia* in raccoons and led to the discovery of two new clades of parasites, bringing the total *Babesia* species diversity in raccoons to four species in the US with an additional fifth species present, to date, only in raccoons from Japan. Our data support the use of *cox1* for the classification of the piroplasms as we obtained the same well supported clades of piroplasms as previous studies and also identified potential geographic seperation of eastern and western parasites in three of the *Babesia* clades from raccoons (Chae et al., 1999; Allsopp and Allsopp, 2006). For several *Babesia* clades, we identified spatial genetic variation which raises interesting questions about *Babesia* transmission and/or raccoon population structure that has led to these spatial variants. Finally, we obtained data that suggests raccoons are the natural host of the *Babesia* sp. detected in a sick maned wolf which highlights the need for tick preventive use and raccoon control around this species and possibly other species of concern.

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TABLE 2.1. Primers used for PCR analyses.

Region	**Primer**	**Sequence (5'-3')**
V4 of 18S	455-479 F	GTCTTGTAATTGGAATGATGGTGAC
V4 of 18S	793-772 R	ATGCCCCCAACCGTTCCTATTA
V4 of 18S	*B*mlike F	CTGCCTTATCATTAATTTCGCTTCCGAACG
cox1	Babcox1 F	GGAAGTGGWACWGGWTGGAC
cox1	Babcox1 R	TTCGGTATTGCATGCCTTG
18S rRNA	5-22 F	GTTGATCCTGCCAGTAGT
18S rRNA	1661 R	AACCTTGTTACGACTTCTC

Table 2.2. Test of significance of prevalence for each location compared to other locations. Values that are significant are highlighted in blue. A) Fisher's exact test for *B. microti*-like sp. infections. B) Fisher's exact test for *Babesia* sensu stricto infections. C) Fisher's exact test for co-infections

A	Florida	West Virginia	Pennsylvania	Nova Scotia	Ontario	Minnesota	Missouri	Texas	Colorado	California
Georgia	0	0.0001	0.1296	0.0001	0.3594	0.0006	0.1785	1	0.3946	0.0001
Florida	X	0.0001	0.0206	0.0001	1	0.0001	0.0441	1	0.1934	0.0001
West Virginia	X	X	0.0048	0.0001	0.0001	0.7769	0.0391	0.2831	0.2261	0.1449
Pennsylvania	X	X	X	0.0001	0.0445	0.0538	1	1	1	0.0001
Nova Scotia	X	X	X	X	0.0001	0.0001	0.0001	0.0006	0.0001	0.0001
Ontario	X	X	X	X	X	0.0009	0.0714	1	0.2051	0.0001
Minnesota	X	X	X	X	X	X	0.1555	0.5296	0.3805	0.0929
Missouri	X	X	X	X	X	X	X	1	1	0.0004
Texas	X	X	X	X	X	X	X	X	1	0.0784
Colorado	X	X	X	X	X	X	X	X	X	0.0205
California	X	X	X	X	X	X	X	X	X	X

B	Florida	West Virginia	Pennsylvania	Nova Scotia	Ontario	Minnesota	Missouri	Texas	Colorado	California
Georgia	0.7752	0.0135	0.0001	0.0001	0.0005	0.0001	0.0409	1	0.0763	0.0539
Florida	X	0.0532	0.0018	0.0001	0.0029	0.0006	0.084	0.2597	0.1332	0.1325
West Virginia	X	X	0.5827	0.0001	0.3517	0.1316	1	1	1	0.7819
Pennsylvania	X	X	X	0.0002	0.7607	0.2658	0.7477	1	1	0.2806
Nova Scotia	X	X	X	X	0.0053	0.2079	0.0004	1	0.0909	0.0001
Ontario	X	X	X	X	X	0.6368	0.4719	1	1	0.2168
Minnesota	X	X	X	X	X	X	0.1884	1	0.4828	0.0691
Missouri	X	X	X	X	X	X	X	1	1	0.7585
Texas	X	X	X	X	X	X	X	X	1	0.5576
Colorado	X	X	X	X	X	X	X	X	X	0.6532
California	X	X	X	X	X	X	X	X	X	X

C	Florida	West Virginia	Pennsylvania	Nova Scotia	Ontario	Minnesota	Missouri	Texas	Colorado	California
Georgia	1	0.001	0.0003	0.0001	0.0011	0.003	0.0055	0.2604	0.1402	0.0017
Florida	X	0.0025	0.0018	0.0001	0.0029	0.0006	0.0144	0.2597	0.1332	0.0043
West Virginia	X	X	1	0.0021	1	0.3932	1	1	1	1
Pennsylvania	X	X	X	0.0002	0.7607	0.2658	1	1	1	1
Nova Scotia	X	X	X	X	0.0053	0.2079	0.01	1	0.0909	0.0017
Ontario	X	X	X	X	X	0.6368	1	1	1	1
Minnesota	X	X	X	X	X	X	0.6086	1	0.4828	0.3863
Missouri	X	X	X	X	X	X	X	1	1	1
Texas	X	X	X	X	X	X	X	X	1	1
Colorado	X	X	X	X	X	X	X	X	X	1
California	X	X	X	X	X	X	X	X	X	X

TABLE 2.3. *Babesia* infections in raccoons by state and infection type. Prevalences are displayed in parentheses.

Country	**Location**	***B. microti*-like sp.**	***Babesia* species sensu stricto**	**Coinfection**
United States	Georgia	151/160 (94%)	74/160 (44%)	70/160 (44%)
	Florida	70/71 (99%)	31/71 (44%)	31/71 (44%)
	West Virginia	21/35 (60%)	8/35 (23%)	5/35 (14%)
	Pennsylvania	47/54 (87%)	9/54 (17%)	9/54 (17%)
	Minnesota	13/21 (62%)	1/21 (5%)	1/21 (5%)
	Texas	3/3 (100%)	0/3 (0%)	0/3 (0%)
	Colorado	7/8 (88%)	1/8 (13%)	1/8 (13%)
	California	13/33 (39%)	9/33 (27%)	5/33 (15%)
Canada	Ontario	31/31 (100%)	4/31 (13%)	4/31 (13%)
	Nova Scotia	5/80 (6%)	0/80 (0%)	0/80 (0%)

Table 2.4. Results of the screening PCR compared to results for the 18S and *Cox1* gene sequencing results. Total samples run for each PCR type are listed in heading (n). Samples with unique sequencing results compared to the screening results are shaded.

Sample ID	State	18 rRNA Screening PCR (n=43)		18S rRNA sequencing (n=17)					*Cox1* sequence results (n=40)				
		B. microti-like sp.	*Babesia* sensu stricto	+/-	*B. microti*-like	*B. lotori*-like	Western group	*Babesia* sp.	+/-	*B. microti*-like	*B. lotori*-like	Western group	*Babesia* sp.
Rac 2	GA	+	+	+	+				-				
Rac 008	GA	+	+	+*					+			+	
Rac 122	GA	+	-	+	+				+			+	
Rac 123	GA	+	+	ND					+	+			
Rac 126	GA	+	+	ND					+	+			
Rac 169	GA	+	+	ND					+	+			
FL 1	FL	+	+	+	+				-				
FL 11	FL	+	-	ND					+	+			
FL 13	FL	+	+	ND					+	+			
FL 16	FL	+	-	ND					+	+			
FL 31	FL	+	+	ND					+	+			
FL 32	FL	+	-	ND					+	+			
WV 3	WV	+	-	ND					+	+			

WV 10	WV	-	+	+				+	+		+		
WV 14	WV	+	-	+	+				+	+			
WV 16	WV	+	+	ND					+		+		
PA-1	PA	+	-	+	+				+				+
PAF-6	PA	+	+	ND					+		+		
PAF-9	PA	+	+	+		+			+		+		
PAF-12	PA	+	+	ND					+		+		
15-7455	MN	+	-	+	+				+				+
15-7986	MN	+	-	ND					+	+			
15-11512	MN	+	-	+*					+	+			
MOI	MO	+	+	ND					+	+			
Rac I	MO	+	+	ND					+	+			
Rac L	MO	+	-	+	+				+	+			
Rac P	MO	-	+	+		+			+		+		
Rac Q	MO	+	+	+*					+		+		
TX 2	TX	+	+	ND					+	+			
W1110-15	ONT	+	+	ND					+	+			

W504-15	ONT	+	+	+*					+	+			
W507-15	ONT	+	+	+				+	+				+
ID 2	ID	-	+	ND					+		+		
CO 8	CO	+	+	+				+	+				+
SM 1	CA	+	+	+*					+			+	
SM 2	CA	-	+	ND					+		+		
SM 3	CA	+	-	+	+				+	+			
Son 4	CA	-	+	+		+			+		+		
SF 2	CA	+	-	ND					+	+			
CC3	CA	+	+	ND					+	+			
Mar 1	CA	+	-	+	+				+*				
Mar 3	CA	+	-	+			+		+			+	
Mar 6	CA	+	+	+*					+			+	

*: Sample amplified with the PCR but sequence failed.

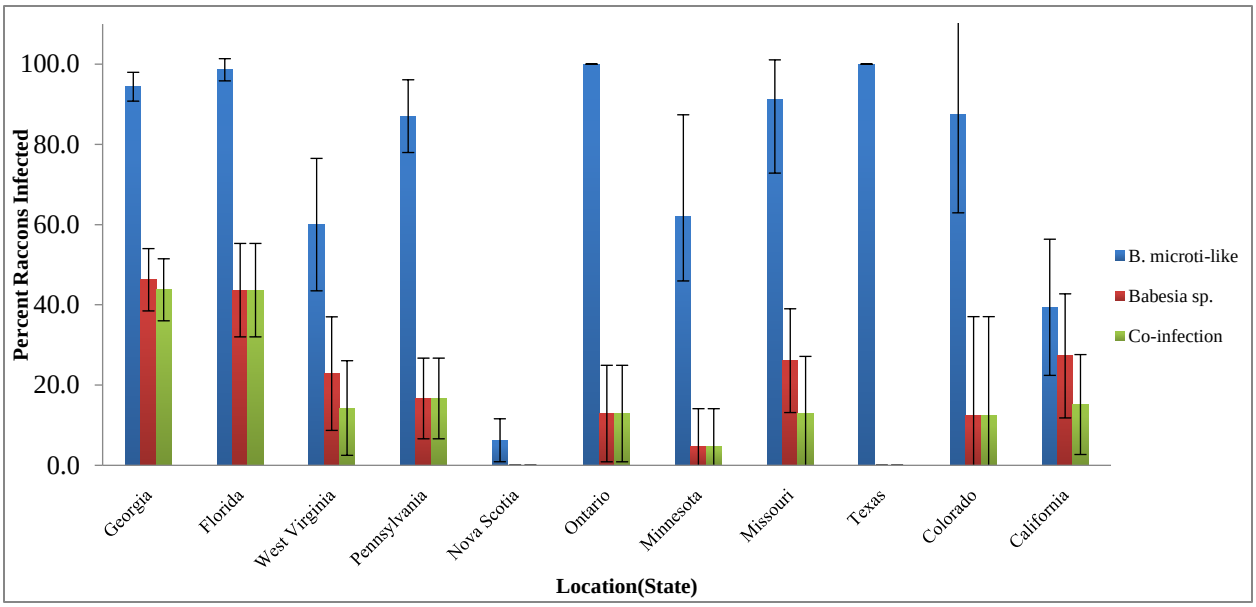

FIGURE 2.1. Prevalence of infection at each location with 95% Confidence Intervals

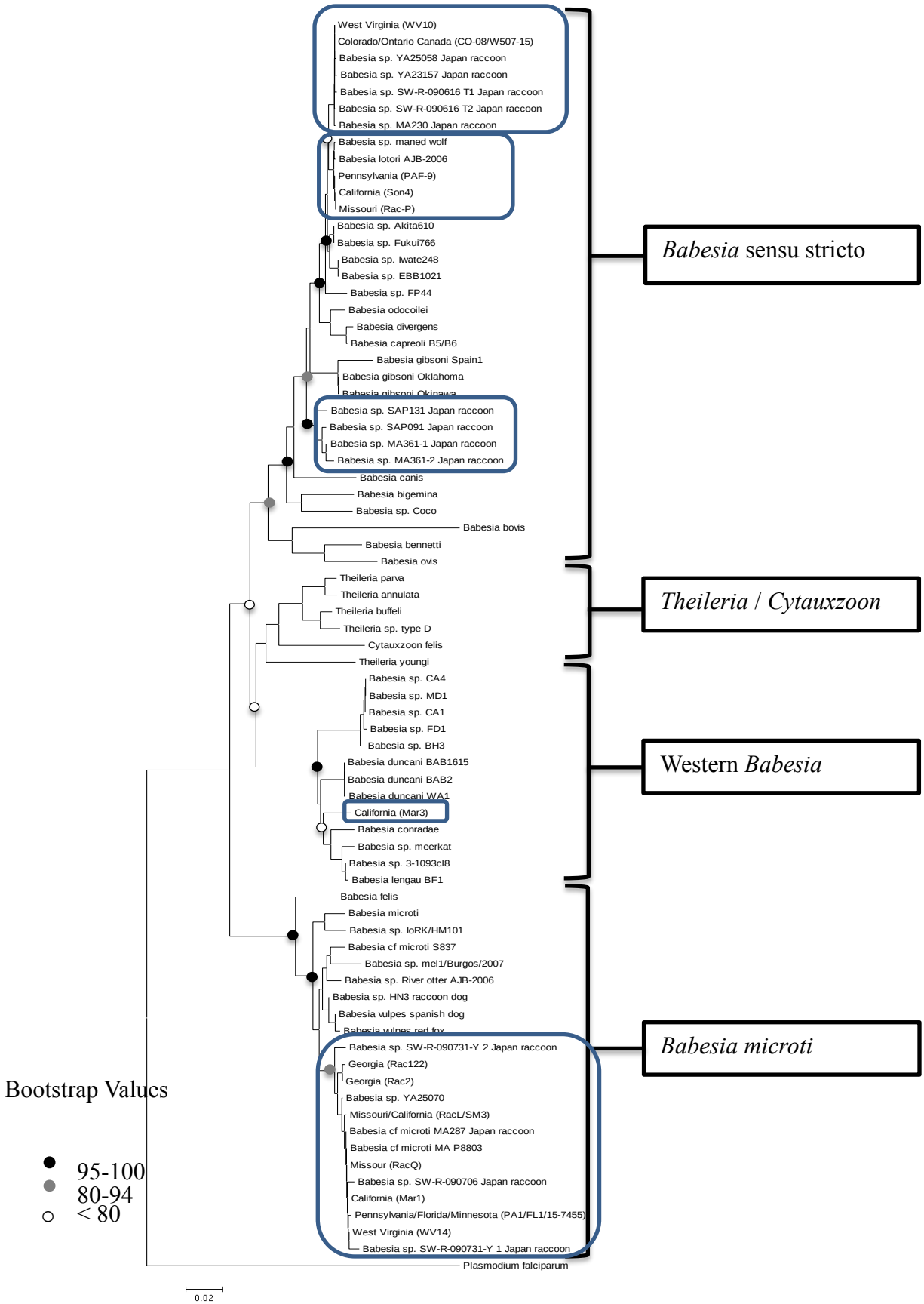
West Virginia (WV10)
Colorado/Ontario Canada (CO-08/W507-15)
Babesia sp. YA25058 Japan raccoon
Babesia sp. YA23157 Japan raccoon
Babesia sp. SW-R-090616 T1 Japan raccoon
Babesia sp. SW-R-090616 T2 Japan raccoon
Babesia sp. MA230 Japan raccoon
Babesia sp. maned wolf
Babesia lotori AJB-2006
Pennsylvania (PAF-9)
California (Son4)
Missouri (Rac-P)
Babesia sp. Akita610
Babesia sp. Fukui766
Babesia sp. Iwate248
Babesia sp. EBB1021
Babesia sp. FP44
Babesia odocoilei
Babesia divergens
Babesia capreoli B5/B6
Babesia gibsoni Spain1
Babesia gibsoni Oklahoma
Babesia gibsoni Okinawa
Babesia sp. SAP131 Japan raccoon
Babesia sp. SAP091 Japan raccoon
Babesia sp. MA361-1 Japan raccoon
Babesia sp. MA361-2 Japan raccoon
Babesia canis
Babesia bigemina
Babesia sp. Coco
Babesia bovis
Babesia bennetti
Babesia ovis
Theileria parva
Theileria annulata
Theileria buffeli
Theileria sp. type D
Cytauxzoon felis
Theileria youngi
Babesia sp. CA4
Babesia sp. MD1
Babesia sp. CA1
Babesia sp. FD1
Babesia sp. BH3
Babesia duncani BAB1615
Babesia duncani BAB2
Babesia duncani WA1
California (Mar3)
Babesia conradae
Babesia sp. meerkat
Babesia sp. 3-1093cl8
Babesia lengau BF1
Babesia felis
Babesia microti
Babesia sp. loRK/HM101
Babesia cf microti S837
Babesia sp. mel1/Burgos/2007
Babesia sp. River otter AJB-2006
Babesia sp. HN3 raccoon dog
Babesia vulpes spanish dog
Babesia vulpes red fox
Babesia sp. SW-R-090731-Y 2 Japan raccoon
Georgia (Rac122)
Georgia (Rac2)
Babesia sp. YA25070
Missouri/California (RacL/SM3)
Babesia cf microti MA287 Japan raccoon
Babesia cf microti MA P8803
Missour (RacQ)
Babesia sp. SW-R-090706 Japan raccoon
California (Mar1)
Pennsylvania/Florida/Minnesota (PA1/FL1/15-7455)
West Virginia (WV14)
Babesia sp. SW-R-090731-Y 1 Japan raccoon
Plasmodium falciparum
0.02
Bootstrap Values
95-100
80-94
< 80
Babesia sensu stricto
Theileria / Cytauxzoon
Western Babesia
Babesia microti

FIGURE 2.2: Phylogenetic analysis of *Piroplasmida* based on the partial 18S rRNA gene using the neighbor joining algorithm. Bootstrap values are denoted with black circles representing a bootstrap support of 95-100%, gray circles representing a bootstrap support of 80-94%, and white circles representing a bootstrap support of 80% or less.

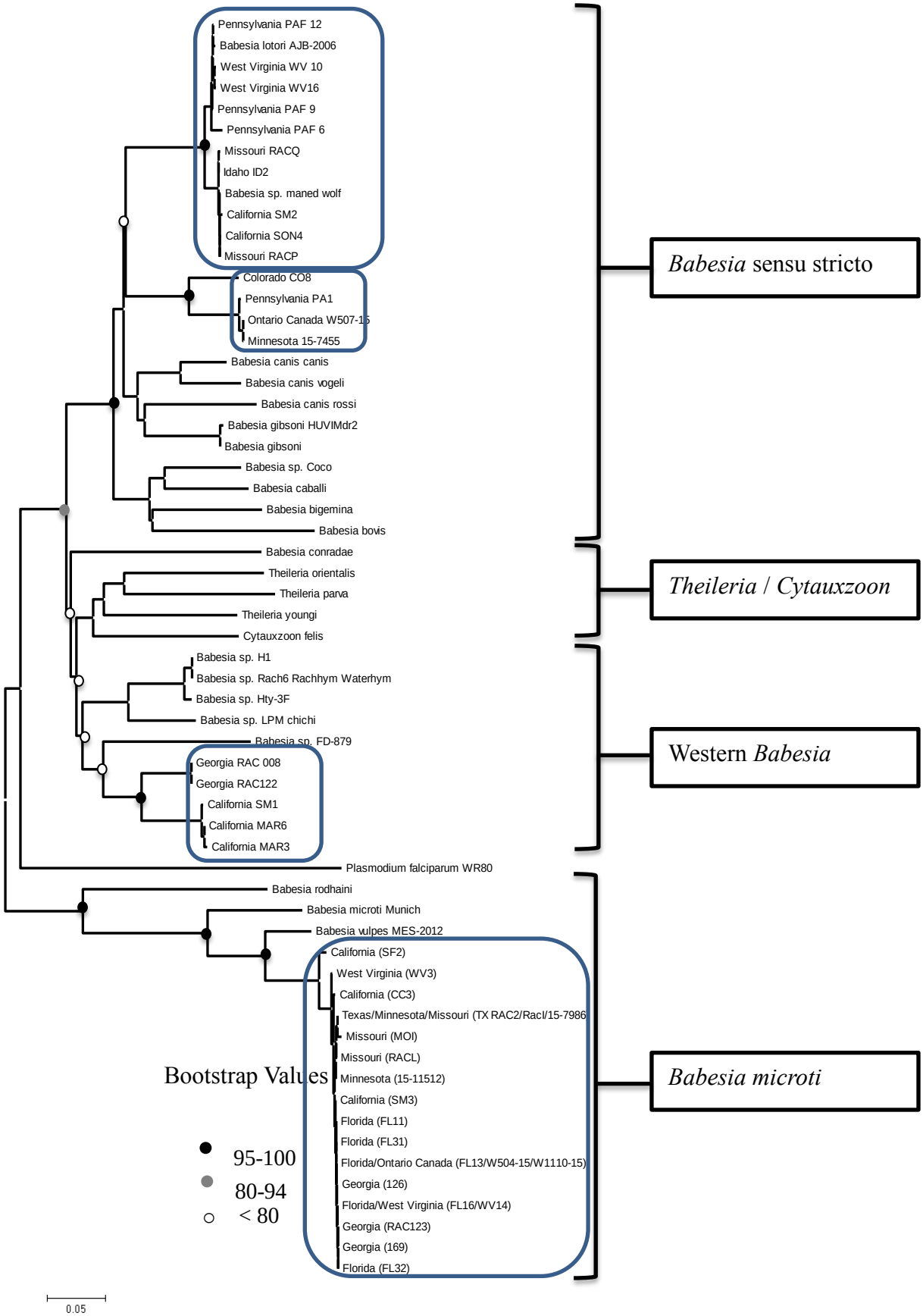
Pennsylvania PAF 12
Babesia lotori AJB-2006
West Virginia WV 10
West Virginia WV16
Pennsylvania PAF 9
Pennsylvania PAF 6
Missouri RACQ
Idaho ID2
Babesia sp. maned wolf
California SM2
California SON4
Missouri RACP
Colorado CO8
Pennsylvania PA1
Ontario Canada W507-15
Minnesota 15-7455
Babesia canis canis
Babesia canis vogeli
Babesia canis rossi
Babesia gibsoni HUVIMdr2
Babesia gibsoni
Babesia sp. Coco
Babesia caballi
Babesia bigemina
Babesia bovis
Babesia conradae
Theileria orientalis
Theileria parva
Theileria youngi
Cytauxzoon felis
Babesia sp. H1
Babesia sp. Rach6 Rachhym Waterhym
Babesia sp. Hty-3F
Babesia sp. LPM chichi
Babesia sp. FD-879
Georgia RAC 008
Georgia RAC122
California SM1
California MAR6
California MAR3
Plasmodium falciparum WR80
Babesia rodhaini
Babesia microti Munich
Babesia vulpes MES-2012
California (SF2)
West Virginia (WV3)
California (CC3)
Texas/Minnesota/Missouri (TX RAC2/RacI/15-7986)
Missouri (MOI)
Missouri (RACL)
Minnesota (15-11512)
California (SM3)
Florida (FL11)
Florida (FL31)
Florida/Ontario Canada (FL13/W504-15/W1110-15)
Georgia (126)
Florida/West Virginia (FL16/WV14)
Georgia (RAC123)
Georgia (169)
Florida (FL32)
Bootstrap Values
95-100
80-94
< 80
0.05
Babesia sensu stricto
Theileria / Cytauxzoon
Western Babesia
Babesia microti

FIGURE 2.3: Phylogenetic analysis of Piroplasmida based on the cox1 gene using the neighbor joining algorithm. Bootstrap values are denoted with black circles representing a bootstrap support of 95-100%, gray circles representing a bootstrap support of 80-94%, and white circles representing a bootstrap support of 80% or less.

CHAPTER 3

MULTIPLE *BABESIA* INFECTIONS IN YOUNG RACCOONS, ASSOCIATION OF SPLENOMEGALY WITH *BABESIA* SENSU STRICTO-INFECTIONS, AND TICK SPECIES FOUND INFESTING YOUNG RACCOONS

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[1]Garrett, Kayla B., Renée Schott, Lea Peshock, and Michael J. Yabsley. 2017. In revision, submitted to *Parasitology Open*

ABSTRACT

Babesia species are intraerythrocytic parasites that are often transmitted by ixodid ticks, but vertical transmission is an alternative route for some species. In the United States, 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 calculated a spleen weight:body weight ratio. 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 juvenile raccoons (eight + weeks old). Raccoons infected with *Babesia* sensu stricto had significantly higher spleen:body weight ratios compared to 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 *I. scapularis* and *Dermacentor variabilis* were also found on raccoons. We report *Babesia* species infections and infestations with several tick species in very young raccoons.

INTRODUCTION

Babesia are an important cause of disease in humans, domestic animals, and some wildlife (Hunfeld et al., 2008; Yabsley and Shock, 2012), although most *Babesia* species in wildlife demonstrate low pathogenicity for their natural host. Most *Babesia* with known life cycles use ixodid ticks as vectors (Hunfeld et al., 2008) although vertical trasnmission has been noted as a possible alternative transmission route for some *Babesia* species (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; Brown et al., 2015; Adaszek et al., 2016; Costa et al., 2016). In addition, fighting and intermixing of individual's 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 United States (Schaffer et al., 1978; Anderson et al., 1981; Telford and Forrester, 1991; Birkenheuer et al., 2006; Birkenheuer et al., 2007; Clark et al., 2012). Thus far little work has been done to investigate whether or not disease occurs, and if so at what intensity, in raccoons infected with *Babesia*. A study in Japan tested raccoons that had splenomegaly, a symptom associated with *Babesia* infections in other organisms, and found that 8% (2/24) had *Babesia* spp. infections; however, no further studies have been conducted on this possible clinical sign (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). Molecular characterization of parasites from raccoons from Florida, Massachusetts, North Carolina, and Illinois has indicated that raccoons can be infected with two species of *Babesia* that are morphologically similar (Geothert and Telford, 2003; Birkenheuer et al., 2006; Birkenheuer et al., 2007; Clark et al., 2012). One is a novel species related to *B. microti*, now called *B. microti*-like species and the other is in the *Babesia* sensu stricto group and is called *B. lotori* (originally described from raccoons from Connecticut: other names include Babesia sp. AJB-2006) (Anderson et al., 1981; Birkenheuer et al., 2007). Without molecular characterization, it is unknown which or both of these species are present in infected raccoons. Much debate has arisen over whether or not the *Babesia microti* clade, unlike the *Babesia* sensu stricto clade, can be

considered true *Babesia* species or a separate species within *Piroplasmida,* giving rise to possible lifestyle differences between these two raccoon *Babesia* species (Lack et al., 2012; Schreeg et al., 2016). Furthermore, the prevalence and distribution of these two raccoon *Babesia* species is poorly known. Outside the US a low prevalence of *B. microti*-like parasites and at least one, possibly two, *Babesia* sensu stricto species, have been reported from raccoons introduced to Japan (Kawabuchi et al., 2005; Jinnai et al., 2009).

Currently, the transmission route is unknown for both *Babesia* spp. of raccoons but it is presumed to be via ixodid ticks as most *Babesia* species are transmitted by ixodid ticks (Uilenberg, 2006; Hunfeld et al., 2008). Raccoons are commonly infested with a several tick species including *Ixodes texanus, I. cookei, I. scapularis, I. 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). Because these two *Babesia* species are found at high prevalences in North Carolina raccoons, the vector is presumed to be a common tick of raccoons as all of the listed tick species are found in North Carolina; however, there is the possibility of alternative transmission routes (Ouellette et al., 1997; Birkenheuer et al., 2006).

Numerous questions remain regarding the natural history of *Babesia* in raccoons, particularly the prevalence and diversity of different *Babesia* species and primary transmission mechanisms. We hypothesize that young raccoons become infected either through vertical transmission or via *I. texanus,* a common tick species on young raccoons which are primarily transmitted among raccoons in the nest (Anderson et al., 1981). Thus, our objectives were to determine the prevalence of *Babesia* in young raccoons under the age of six weeks old and, if they occured, what species of *Babesia* were present. In addition, we examined young raccoons to

determine if ticks infested young raccoons within the nest, as raccoons younger than six weeks old typically do not venture from the nest (Schneider et al., 1971; Gehrt and Fritzell, 1998). We sampled young raccoons in Minnesota and Colorado where we have previously noted *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 centers 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; Guglielmone et al., 2014).

Data collected from each raccoon included weight, body length, sex, and estimated age based on tooth eruption (Montgomery, 1964). Spleens were removed after examination, weighed, and re-frozen at -20 C until testing. 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 (to compare with aging by tooth eruption) 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 ~10mg of spleen using a commercial kit per manufacture'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; Birkenheuer et al., 2007). One set of primers, BMlikeF (5'-CTGCCTTATCATTAATTTCGCTTCCGAACG) and 793-772R (5'-ATGCCCCCAACCGTTCCTATTA), targets *Babesia* parasites in the *B. microti*-like clade. A BioRad DNA Engine Peltier Thermal Cycler (Bio-Rad Laboratories Incorporated, Foster City, CA) was used and Cycling parameters were 94 C for 5 minutes followed by 49 cycles of 94 C for 45 sec, 56 C for 45 sec, and 72 C for 45 sec, with a final extension of 72 C for 5 minutes. 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 minutes followed by 44 cycles of 94 C for 30 sec, 60 C for 30 sec, 72 C for 30 sec, with a final extension of 72 C for 5 minutes.

Precautions were taken to prevent contamination, including 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 to detect if contamination occurred. 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 sequenced-confirmed presence of *B. lotori* and *B. microti*-like sp.) were included. Amplicons were observed in a GelRed stained 1.5% agarose gel.

Because the *Babesia* sensu stricto PCR protocol amplifies numerous *Babesia* species and is a small amplicon not ideal for species identification, samples positive with this 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 minutes followed by 45 cycles of 95 C for 20 seconds, 50 C for 30 seconds, 68 C for 1 minutes and 30 seconds, and a final extension of 72 C for 5 minutes (Schreeg et al., 2016). 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 Geneious program (Biomatters Limited, Auckland New, Zealand) and consensus sequences compared to other *Babesia* sequences in GenBank.

Some *Ixodes* ticks were damaged during removal and could not be identified to species using morphology and thus were assigned to species based on molecular methods. 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 minutes

followed by 40 cycles of 94 C for 30 seconds, 45 C for 30 seconds, 72 C for 1 minute, and a final extension of 72 C for 5 minutes. Amplicons were sequenced as described above.

Statistical analyses

A Fisher's exact test was used to compare the overall prevalence of the *Babesia microti*-like sp. infections and *Babesia* sensu stricto infections. A generalized linear model (GLM) was utilized and the data log transformed to determine if infection with a *Babesia* parasite was related to multiple variables, including sex, age (by week), spleen:body weight ratio, and whether or not ticks were present. A separate GLM was performed for the *Babesia microti*-like sp. and *Babesia* sensu stricto data sets.

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)]. Eight (7.5%) raccoons had co-infections. For the *Babesia microti*-like sp., infections were detected in individuals as young as one week of age and prevalence was high in all age groups (Table 3.1). For the *Babesia* sensu stricto group, infections were first noted at two weeks of age but prevalence was low, while in raccoons six weeks or older prevalence was 40% (Table 3.1). 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 (99.6%) to a *Babesia* sp. reported from a captive maned wolf (*Chrysocyon brachyurus*) (KR017881) but was also 98.1% similar to *Babesia lotori* (accessioned as *Babesia* sp. AJB-2006, KR017882) in raccoons. Two other sequences were identical to each other and,

although they were most similar to *B. lotori*, they only had 83.9% similarity. The remaining two sequences were most similar (90.2%) to a *B. vulpes* (a *B. microti*-like sp. of fox and dogs, accessed in GenBank as *Babesia* sp. MES-2012, KC207827), although 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 co-infected based on 18S PCR.

Using GLM, the only significant variable for raccoons infected with *Babesia 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), indicating that infections occurred more in older individuals and individuals with infections were more likely to have larger spleen:body weight ratios. Also, raccoons with *Babesia* sensu stricto infections or with co-infections of both groups of *Babesia* had higher spleen:body weight ratios compared to raccoons infected with *Babesia microti*-like sp. only or those with no *Babesia* infection (Figure 3.1).

Three tick species were found on young raccoons including *Ixodes texanus, Ixodes scapularis,* and *Dermacentor variabilis*, all from raccoons from Minnesota (Table 3.2). Some ticks were damaged during removal and could not be identified to species, but 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 two weeks of age and the infestation rate was similar for all age groups (Figure 3.2a). In contrast, *I. scapularis* infestations were primarily noted in raccoons older than five weeks of age, although a single three-week-old raccoon was infested with two *I. scapularis* nymphs (Figure 3.2a). Infestation rates for *D. variabilis* increased with age (Figure 3.2a). The mean number of *I. texanus* collected from infested raccoons was highest for raccoons in the two

week age group with two individuals having 54 and 64 ticks respectively (Figure 3.2b). Other than those two raccoons with high *I. texanus* infestation rates, 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 (Figure 3.3) although two raccoons had ticks present on multiple parts of the body. Presence of ticks was not a significant variable for infection with either *Babesia* species (*Babesia microti*-like sp.: p=0.2393; *Babesia* sensu stricto: p=0.3604).

DISCUSSION

We detected *Babesia* infections in young raccoons from Minnesota and Colorado with the *B. microti*-like sp. detected in individuals as young as one 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, they were much less common. We had sequence confirmation for *Babesia* sensu stricto species closely related to the two previously reported *Babesia* spp. from raccoons, but we also found a possible novel *Babesia* sp. similar to *Babesia* from a maned wolf and *B. lotori*. We also noted co-infections occurring in some young raccoons; however, these infections seemed to be much lower than that of other studies and are likely due to the low number of *Babesia* sensu stricto infections (Birkenheuer et al., 2006). These data extend the known range of *Babesia* infections of raccoons to Colorado and confirm that both *B. microti*-like and *Babesia* sensu stricto occur in Minnesota and Colorado.

In juvenile raccoons, the only previous study to investigate *Babesia* was conducted in Connecticut and our data support those findings (Anderson et al., 1981); however, infections in raccoons were determined in that study based on blood smear analysis and thus the species of *Babesia* present was unknown. Anderson et al. (1981) found that three of four young raccoons that were still confined to a nest in a chimney were positive for *Babesia* infections, and nymphal *Ixodes texanus* ticks were found on two of the raccoons. Unfortunateley, the age of the raccoons was not given.

Our primary goal 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 rate of tick infestation on raccoons from Minnesota, it is unknown if the infections in young raccoons were acquired vertically from infected female raccoons or due to infestation with ticks at a very young age. While we did not find ticks on raccoons younger than two weeks of age, our sample size for one-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 the mother was not available for analysis.

The prepatent period is generally unknown for many *Babesia* species and varies by transmission route and detection method, so reported data may not be valid for raccoon-infecting *Babesia* species (Gumber et al., 2016). Inoculation of rhesus macaques, simulating tick transmission of *Babesia*, with *B. microti*-infected blood resulted in parasitemia in 4 days for monkey-passaged parasites vs. 35 days for hamster-passaged parasites (Gumber et al., 2016).

For vertical transmission of *Babesia microti*, voles in Europe have prepatent periods noted at three weeks of age, and experimentally infected BALB rodents after 20 days (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 *Babesia microti*-like sp. infections in raccoons as young as 1 week old, which is on the low end of known prepatent periods for transmission and is generally lower than prepatent periods associated with tick transmission (Tolkacz et al., 2017).

For *Babesia* sensu stricto spp., studies investigating prepatent periods after exposure of hosts to infected ticks found that the prepatent period for *B. canis* transmitted to dogs by *Rhipicephalus sanguineus* was six days post infection whereas cattle became infected with *Babesia major* within 9-15 days after exposure to infected *Haemaphysalis punctate*, and beagle puppies experimentally infected with *Babesia gibsoni* had detectible parasites 15 days after inoculation (Paraense, 1949; Yin et al., 1996; Brown et al., 2015). Vertical transmission of *Babesia* sensu stricto sp. has also been noted with beagle puppies, whose mother was intravenously inoculated with *Babesia gibsoni* prior to mating, showing infection after 14 days (Fukumoto et al., 2005). Other cases of vertical transmission of *Babesia* sensu stricto sp. were seen in Central Asian Shepherd dogs, where the puppies were found to have *Babesia canis* infections six weeks after birth, and in Russian Terriers, where the puppies showed clinical infections after eight weeks from possible vertical transmission of *Babesia canis canis* (Mierzejewska et al., 2014; Adaszek et al., 2016). Because infections of raccoons with *Babesia* sensu stricto were not noted until at least two to three weeks of age, which is within the time frame of tick-transmitted prepatent periods, it is possible that these two groups of *Babesia*, *Babesia microti*-like sp. and *Babesia* sensu stricto sp., utilize different transmission strategies.

Ticks, specifically ixodid ticks, are the main vector for many *Babesia* sp. and are important to note when considering the lifecycle of these raccoon *Babesia* species (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 hosts 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 and is found throughout the entire United States from North Carolina, to Alaska and California (Ouellette et al., 1997; Gabriel et al., 2009; Durden et al., 2016). The other two species found on our raccoons included *D. variablis,* which is restricted to the Eastern US and in isolated populations in California, and *I. scapularis,* which is restricted to the Eastern US (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 *Babesia microti*, the primary cause of human babesiosis in the US (Hersh et al., 2012). Raccoons younger than six to eight weeks most likely acquire ticks from the mother, as young of this age do not typically venture from the nest, while older individuals (seven to ten week old young) are more active and can become infested with ticks outside of the nest (Schneider et al., 1971; Gehrt and Fritzell, 1998). 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).

Babesia 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 have 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 *Babesia lotori* (Birkenheuer et al., 2006). The raccoon was found nonambulatory with pronounced anemia, hypoproteinemia, hypalbuminemia, and elevated alanine aminotransferase with rare intraerythrocytic *Babesia* parasites. It was treated for *Babesia* and released; however it is unkown 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 *Babesia* 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 was collected nor was any histologic analysis done to determine 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.

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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TABLE 3.1. Prevalence of *Babesia* spp. in young raccoons from Colorado and Minnesota, by age class[a]

		Age in Weeks No. positive/No. tested (%positive)							
State	Parasite	<1[b]	1	2	3	4	5	6+	Total
Minnesota	*Babesia microti*-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)
	Babesia s.s. group	0/3	0/3	0/19	3/25 (12)	1/18 (6)	1/5 (20)	3/10 (30)	8/83 (10)
	Co-infected	0/3	0/3	0/19	2/25 (8)	0/18	0/5	3/10 (30)	5/83 (6)
Colorado	*Babesia microti*-like sp.	NT	3/3 (100)	1/3 (33)	1/3 (33)	NT	3/3 (100)	4/5 (80)	12/17 (70)
	Babesia s.s. group	NT	0/3	1/3 (33)	0/3	NT	0/3	2/5 (40)	3/17 (18)
	Co-infected	NT	0/3	1/3 (33)	0/3	NT	0/3	2/5 (40)	3/17 (18)
Total	*Babesia microti*-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)
	Babesia s.s. group	0/3	0/3	1/22 (5)	3/28 (11)	1/18 (6)	1/8 (13)	5/15 (33)	11/100 (11)
	Co-infected	0/3	0/3	1/22 (5)	2/28 (7)	0/18	0/8	5/15 (33)	8/100 (8)

[a]Age was not available for 6 raccoons

[b]these three raccoons were near term and removed via caesarian section from a deceased female.

TABLE 3.2. Number and Stage of ticks collected from young raccoons from Minnesota

		Tick Stage			
Tick Species	n	Larvae	Nymph	Male	Female
Ixodes texanus	211	7	133	1	70
Ixodes scapularis	9	3	6	0	0
Dermacentor variabilis	49	0	1	18	30

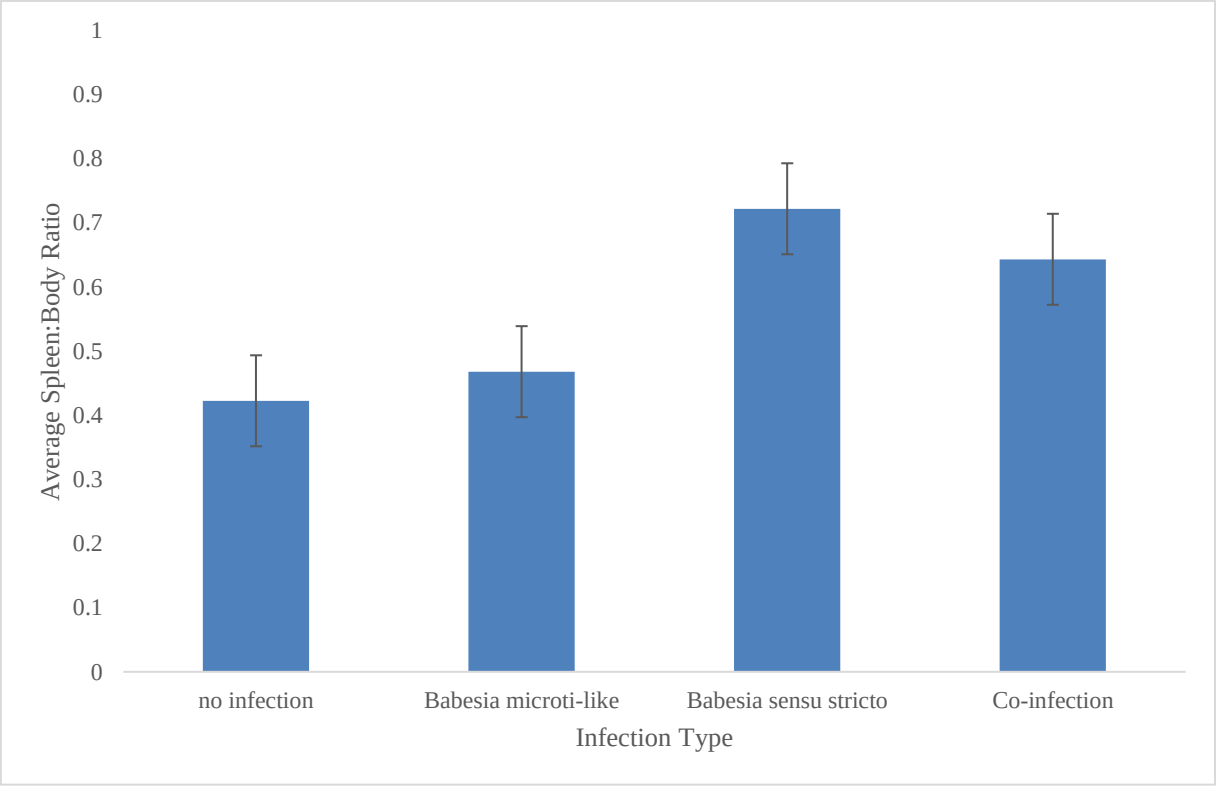

FIGURE 3.1. Effects of *Babesia* infection on the average spleen:body weight ratio of young raccoons with standard error.

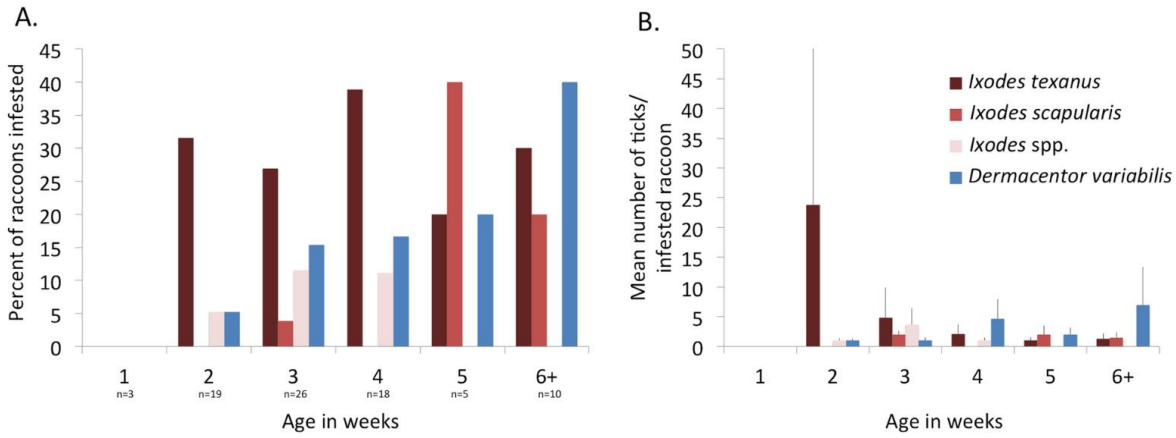

FIGURE 3.2. A. Percent of young raccoons infested with ticks by age class in weeks (number of raccoons sampled in each age class shown below age). B. Average number of ticks from infested raccoons in each age class in weeks. Number of raccoons sampled is same as in A.

FIGURE 3.3. Ticks on a 1.5 week old raccoon. A. An adults *Dermacentor variabilis* on snout. B. Several nymphal and adult *Ixodes texanus* in an ear. C. Several adult *Ixodes texanus* in an ear.

CHAPTER 4

CONCLUSIONS

Babesia spp. are an incredibly diverse group of parasites, many of which are of veterinary and medical importance. Historically, *Babesia* infections in raccoons were considered to be asymptomatic and of little clinical relevance to other host species. For this reason, raccoon (*Procyon lotor*) *Babesia* infections have not been the subject of many research projects. The objectives of this study were to provide a better understanding of the prevalence, distribution, and diversity of *Babesia* spp. in raccoons in selected areas of the United States and parts of Canada. Knowledge of the range of *Babesia* in raccoons may have provided insight into a possible tick vector as the transmission routes of these *Babesia* spp. is unknown. We also wanted to investigate the intraspecific variation for these *Babesia* spp. and determine if there was spatial associations with genetic variants. Finally we investigated *Babesia* infections in young raccoons to determine if infections were occurring and, if so, at what rate. We also evaluated what tick species were infesting young raccoons and if any clinical disease was associated with *Babesia* infections in young raccoons.

The purpose of this study was to evaluate the prevalence and distribution of *Babesia* species in raccoons throughout the United States and Canada, as well as evaluate if any intraspecific variation was occurring within each *Babesia* species, such as geographic variation. *Babesia* infections were noted in high prevalences throughout the United States and Canada,

with the highest prevalences being in the southeastern United States (*B. microti*-like sp. [60-100%] *B.* sensu stricto [20-40%]). *Babesia microti*-like sp. infections were higher than those of *Babesia* "sensu stricto" species, and co-infections were common in areas with *B.* "sensu stricto" species. Phylogenetic analyses revealed that possibly multiple piroplasms exist in North America, with two possible species in the *Babesia* sensu stricto group (*Babesia lotori* and a novel *Babesia* sensu stricto sp.), one in the Western *Babesia* group, and a *Babesia microti*-like species unique to raccoons. We also noted possible geographic variation in both the *Babesia lotori* group and the novel Western piroplasms species, with eastern and western sequences grouping separately. More research is needed to truly classify these piroplasms in raccoons and determine how many species really exist as well as evaluate the molecular protocols in place for diagnosing infections in raccoons, as they may not be as specific as previously thought.

In the study on young raccoons, the main goal was to determine if infections occurred and to speculate if vertical transmission was plausible for *Babesia* infections in raccoons. Infections were evaluated using PCR assays to determine what species of *Babesia* may be present. Tick species that were found on raccoons were identified either using a key or with PCR. Finally, the study speculated on whether or not any clinical signs of *Babesia* infections were present based on the morphological data collected from the raccoons, such as age, weight, spleen weight, and sex. The results showed that both known species of *Babesia* were present in young raccoons, with a third yet unknown species also present. *Babesia microti*-like infections were common and *Babesia* sensu stricto infections less so, with co-infections also occurring. Morphologic data was evaluated with infections using a generalized linear model and showed that age was a significant variable for infections with both *B. microti*-like sp. and *B.* sensu-stricto; however, *B.* sensu stricto infections were also correlated with larger spleens indicating

that splenomegaly may be associated with *B.* sensu stricto infections. For *B. microti*-like sp. in young raccoons, infections were noted in raccoons as young as one-week old indicating that vertical transmission could be possible; however, the prepatent period for *Babesia* in raccoons is unknown and further investigation into this method of transmission is still required. Infections with *B.* sensu stricto were typically in older raccoons, indicating that tick transmission is more likely. Multiple tick species were found infesting young raccoons, including *Ixodes texanus, I. scapularis,* and *Dermacentor variabilis*, with the most common tick species being *Ixodes texanus.*

The results of these studies verify that *Babesia* infections are occurring in raccoons throughout regions of North America. Phylogenetic analyses of these piroplasms indicate that raccoon *Babesia* species are far more diverse than previously thought, and further analyses are needed to evaluate the actual nature of these infections in raccoons. Future research should focus on testing the *cox1* region of the Japanese samples in Genbank to better evaluate how many species can infect raccoons within the *Babesia* sensu stricto group. Further analysis of this new Western *Babesia* species in raccoons is also needed to characterize this novel species. We also recommend that an evaluation of the taxonomic classification of the *Babesia microti* and *Babesia microti*-like species in carnivores be conducted in future studies as it is evident that this group, and possibly others, are not true *Babesia*.

APPENDIX

PERCENT IDENTITY TABLES FOR THE 18S AND *COX1* ANALYSES

Number	Sample ID
1	California_(Mar3)
2	Babesia_conradae
3	Babesia_sp._meerkat
4	Babesia_duncani_WA1
5	Babesia_duncani_BAB1615
6	Babesia_duncani_BAB2
7	Babesia_sp._3-1093cl8
8	Babesia_sp._CA4
9	Babesia_sp._MD1
10	Babesia_sp._BH3
11	Babesia_sp._CA1
12	Babesia_sp._FD1
13	Babesia_lengau_BF1
14	Babesia_sp._maned_wolf
15	Babesia_lotori_AJB-2006
16	California_(Son4)
17	Pennsylvania_(PAF-9)
18	Missouri_(Rac-P)
19	West_Virginia_(WV10)
20	Colorado/Ontario_Canada_(CO-08/W507-15)
21	Babesia_sp._YA23157_Japan_raccoon
22	Babesia_sp._SW-R-090616_T1_Japan_raccoon
23	Babesia_sp._SW-R-090616_T2_Japan_raccoon
24	Babesia_sp._YA25058_Japan_raccoon
25	Babesia_sp._MA230_Japan_raccoon
26	Babesia_sp._SAP091_Japan_raccoon
27	Babesia_sp._MA361-1_Japan_raccoon
28	Babesia_sp._MA361-2_Japan_raccoon
29	Babesia_sp._SAP131_Japan_raccoon
30	Babesia_odocoilei
31	Babesia_divergens
32	Babesia_bennetti
33	Babesia_bovis
34	Babesia_ovis
35	Babesia_bigemina
36	Babesia_canis
37	Babesia_gibsoni_Oklahoma
38	Cytauxzoon_felis
39	Theileria_parva
40	Theileria_annulata
41	Theileria_buffeli
42	Theileria_sp._type_D
43	Theileria_youngi
44	Babesia_microti
45	Babesia_felis
46	Babesia_cf_microti_S837
47	Babesia_sp._River_otter_AJB-2006
48	Babesia_cf_microti_MA287_Japan_raccoon
49	California_(Mar1)
50	Georgia_(Rac122)
51	Missour_(RacQ)
52	Missouri/California_(RacL/SM3)
53	Georgia_(Rac2)
54	West_Virginia_(WV14)
55	Pennsylvania/Florida/Minnesota_(PA1/FL1/15-7455)
56	Babesia_cf_microti_MA_P8803
57	Babesia_sp._SW-R-090706_Japan_raccoon
58	Babesia_sp._SW-R-090731-Y_2_Japan_raccoon
59	Babesia_sp._SW-R-090731-Y_1_Japan_raccoon
60	Babesia_sp._YA25070
61	Babesia_vulpes_spanish_dog
62	Babesia_vulpes_red_fox
63	Babesia_sp._HN3_raccoon_dog
64	Babesia_sp._mel1/Burgos/2007
65	Babesia_sp._IoRK/HM101
66	Babesia_sp._Akita610
67	Babesia_gibsoni_Spain1
68	Babesia_gibsoni_Okinawa
69	Babesia_sp._Fukui766
70	Babesia_sp._Coco
71	Babesia_sp._Iwate248
72	Babesia_sp._FP44
73	Babesia_sp._EBB1021
74	Babesia_capreoli_B5/B6
75	Plasmodium_falciparum

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59
1	X																																																												
2	99.9	X																																																													
3	99.5	99.6	X																																																											
4	99.6	**99.8**	99.9	X																																																										
5	99.9	100.0	99.6	99.8	X																																																									
6	98.0	98.1	97.8	97.9	98.1	X																																																							
7	99.4	99.5	99.6	99.8	99.5	98.1	X																																																						
8	99.8	99.9	99.6	99.8	99.9	98.2	99.5	X																																																					
9	100.0	100.0	99.6	99.7	100.0	98.2	99.5	99.9	X																																																				
10	99.4	99.5	99.9	99.7	99.5	**97.7**	99.5	99.5	99.6	X																																																			
11	99.6	99.8	99.4	99.5	99.8	98.1	99.5	99.6	99.7	99.2	X																																																
12	99.6	99.8	99.9	100.0	99.8	97.9	99.8	99.8	99.7	99.7	99.5	X																																															
13	99.9	100.0	99.6	99.8	100.0	98.1	99.5	99.9	100.0	99.5	99.8	99.8	X																																														
14	99.9	100.0	99.6	99.8	100.0	98.1	99.5	99.9	100.0	99.5	99.8	99.8	100.0	X																																													
15	99.9	100.0	99.6	99.8	100.0	98.1	99.5	99.9	100.0	99.5	99.8	99.8	100.0	100.0	X																																												
16	51.2	51.5	51.4	51.5	51.4	53.0	51.4	51.6	51.1	51.0	51.9	51.4	51.4	51.4	51.4	X																																												
17	50.5	50.8	50.8	50.8	50.8	52.1	50.8	50.9	50.4	50.3	51.2	50.8	50.7	50.7	50.8	98.7	X																																											
18	53.9	53.8	54.3	54.0	53.9	54.8	53.7	54.1	53.5	53.4	53.9	54.1	53.6	53.6	53.9	80.1	79.8	X																																										
19	53.5	53.3	53.7	53.3	53.5	54.3	53.0	53.6	53.0	52.7	53.5	53.5	53.2	53.2	53.5	80.0	79.6	99.9	X																																									
20	48.2	48.2	48.6	48.7	48.2	47.5	48.2	48.1	46.8	46.9	48.2	48.6	48.1	48.1	48.2	71.5	71.7	72.4	72.3	X																																								
21	51.4	51.5	51.9	51.7	51.4	51.4	51.2	51.8	51.6	51.7	51.4	51.7	51.4	51.4	51.4	77.0	76.7	78.6	78.6	70.8	X																																							
22	53.6	53.5	54.1	53.7	53.6	54.5	53.4	53.8	53.2	53.1	53.6	53.9	53.4	53.4	53.6	80.1	79.8	99.6	99.5	72.6	79.0	X																																							
23	99.9	100.0	99.6	99.8	100.0	98.1	99.5	99.9	100.0	99.5	99.8	99.8	100.0	100.0	100.0	51.4	50.8	53.9	53.5	48.2	51.4	53.6	X																																							
24	51.2	51.5	51.4	51.5	51.4	53.0	51.4	51.6	51.1	51.0	51.9	51.4	51.4	51.4	51.4	100.0	98.7	80.1	80.0	71.5	77.0	80.1	51.4	X																																							
25	54.3	54.6	54.5	54.6	54.5	55.1	54.5	54.2	53.9	53.6	54.9	54.5	54.4	54.4	54.5	81.2	81.3	90.4	90.2	72.9	79.6	90.4	54.5	81.2	X																																							
26	54.3	54.6	54.5	54.6	54.5	55.1	54.5	54.2	53.9	53.6	54.9	54.5	54.4	54.4	54.5	81.2	81.3	90.4	90.2	72.9	79.6	90.4	54.5	81.2	100.0	X																																							
27	55.4	55.2	54.9	54.8	55.4	55.4	54.9	55.1	56.0	55.0	55.6	54.9	55.1	55.1	55.4	74.6	73.9	73.4	73.5	70.0	71.7	73.6	55.4	74.6	75.4	75.4	X																																							
28	53.3	53.4	53.6	53.4	53.3	54.6	53.3	53.5	53.1	53.1	53.6	53.3	53.3	53.3	53.3	88.7	88.8	79.2	79.0	70.3	76.0	79.1	53.3	88.7	80.1	80.1	73.7	X																																						
29	52.6	52.7	53.1	52.7	52.8	53.7	52.8	52.5	52.7	52.3	53.3	52.8	52.5	52.5	52.8	76.7	76.9	75.4	74.9	70.2	70.4	75.3	52.8	76.7	77.1	77.1	71.8	76.2	X																																						
30	53.2	53.3	53.6	53.3	53.4	54.3	53.4	53.1	53.2	52.9	53.9	53.4	53.1	53.1	53.4	76.7	76.5	75.7	75.2	70.5	69.9	75.6	53.4	76.7	76.8	76.8	72.3	76.0	97.9	X																																					
31	53.4	53.5	53.9	53.5	53.6	54.5	53.6	53.3	53.2	52.9	54.1	53.6	53.4	53.4	53.6	76.4	76.2	75.4	74.8	70.0	69.5	75.2	53.6	76.4	76.5	76.5	71.7	76.0	97.6	99.5	X																																					
32	53.0	53.1	53.4	53.1	53.2	54.1	53.2	52.9	53.0	52.7	53.6	53.2	52.9	52.9	53.2	76.4	76.2	75.4	74.8	70.2	69.9	75.2	53.2	76.4	76.5	76.5	71.9	75.7	97.7	99.6	99.6	X																																					
33	53.0	53.1	53.4	53.1	53.2	54.1	53.2	52.9	53.0	52.7	53.6	53.2	52.9	52.9	53.2	76.7	76.5	75.7	75.2	70.5	69.9	75.6	53.2	76.7	76.8	76.8	72.3	76.0	98.0	99.9	99.6	99.8	X																																					
34	52.5	52.6	53.0	52.6	52.8	53.6	52.8	52.5	52.5	52.2	53.2	52.8	52.5	52.5	52.8	76.8	77.0	75.7	75.2	70.3	70.7	75.6	52.8	76.8	77.3	77.3	72.1	76.3	99.9	98.0	97.8	97.9	98.1	X																																					
35	52.5	52.5	52.9	52.5	52.7	53.6	52.7	52.4	53.0	52.7	53.1	52.7	52.4	52.4	52.7	76.6	76.8	75.0	74.5	69.9	70.3	74.9	52.7	76.6	77.0	77.0	71.7	76.1	99.6	97.7	97.5	97.6	97.9	99.5	X																																					
36	52.0	52.1	52.4	52.1	52.2	53.1	52.2	51.9	52.6	52.3	52.7	52.2	51.9	51.9	52.2	76.0	76.1	74.5	73.9	69.1	69.4	74.3	52.2	76.0	76.3	76.3	71.1	75.4	99.1	97.1	96.9	97.0	97.2	98.8	98.8	X																																					
37	52.8	52.8	53.2	52.8	53.0	53.9	53.0	52.7	53.0	52.7	53.4	53.0	52.7	52.7	53.0	76.7	76.8	75.1	74.5	70.0	70.4	74.9	53.0	76.7	77.0	77.0	71.7	76.2	99.6	97.8	97.5	97.6	97.9	99.5	99.8	98.7	X																																					
38	51.4	51.2	51.8	51.7	51.4	52.7	51.8	51.7	50.7	50.3	51.6	51.8	51.1	51.1	51.4	73.5	73.5	75.6	75.4	69.5	71.5	75.6	51.4	73.5	76.1	76.1	72.3	74.3	82.1	81.4	81.4	81.3	81.6	82.0	81.7	81.5	81.8	X																																					
39	51.7	51.5	51.7	51.5	51.7	52.3	52.1	51.8	51.4	50.5	52.1	51.7	51.4	51.4	51.7	72.4	72.7	74.2	73.8	68.9	69.6	74.4	51.7	72.4	73.7	73.7	70.0	72.8	83.4	82.5	82.2	82.3	82.6	83.1	83.0	83.3	83.2	91.2	X																																					
40	53.2	53.3	53.6	53.3	53.4	54.3	53.4	53.1	53.2	52.9	53.9	53.4	53.1	53.1	53.4	76.5	76.4	75.5	75.0	70.3	69.7	75.4	53.4	76.5	76.7	76.7	72.1	75.8	97.9	99.8	99.8	99.9	99.9	98.0	97.7	97.1	97.8	81.4	82.5	X																																					
41	51.4	51.3	51.4	51.3	51.4	51.7	51.9	51.6	51.4	50.5	51.9	51.4	51.2	51.2	51.4	72.2	72.6	73.9	73.5	68.2	69.5	74.0	51.4	72.2	73.6	73.6	69.8	72.8	83.3	82.3	82.0	82.2	82.5	82.9	82.9	83.2	83.1	91.1	99.5	82.3	X																																					
42	51.6	51.4	51.6	51.4	51.6	51.8	52.0	51.7	51.4	50.5	52.0	51.6	51.3	51.3	51.6	72.2	72.5	73.8	73.4	68.1	69.4	74.0	51.6	72.2	73.5	73.5	69.8	72.8	83.3	82.3	82.0	82.1	82.5	82.9	82.9	83.2	83.0	91.1	99.5	82.3	100.0	X																																				
43	90.3	90.4	90.4	90.6	90.4	90.4	90.6	90.4	90.5	90.5	90.4	90.6	90.4	90.4	90.4	53.0	52.5	55.2	55.0	50.9	54.3	55.6	90.4	53.0	55.6	55.6	54.3	54.6	55.0	55.6	55.4	55.1	55.4	54.9	55.1	54.6	55.1	54.2	54.5	55.1	54.1	54.2	X																																			
44	81.0	81.2	81.0	80.9	81.2	81.0	80.9	81.3	81.0	80.6	81.5	80.9	81.2	81.2	81.2	56.2	55.6	55.4	55.4	50.5	54.3	55.8	81.2	56.2	58.3	58.3	58.1	55.9	56.3	56.4	56.2	56.0	56.2	56.2	56.1	55.9	56.2	55.7	54.5	56.0	54.7	54.9	83.2	X																																			
45	62.4	62.5	63.0	62.9	62.4	62.6	63.0	62.4	64.2	64.5	62.8	62.8	62.5	62.5	62.4	58.5	58.7	60.0	59.6	56.3	60.3	60.3	62.4	58.5	58.7	58.7	58.5	60.0	59.6	59.1	58.7	58.9	58.9	59.3	58.8	59.6	58.9	58.4	58.9	58.7	58.7	58.8	64.9	65.3	X																																			
46	52.3	52.2	52.3	52.2	52.3	53.9	52.8	52.7	51.8	51.2	52.8	52.3	52.0	52.0	52.3	73.6	73.4	73.6	73.3	68.9	70.9	73.9	52.3	73.6	74.9	74.9	70.9	75.0	79.9	80.7	80.9	80.5	80.9	80.1	79.8	79.3	79.8	78.0	77.6	80.7	77.6	77.6	55.6	54.1	54.5	X																																			
47	51.9	51.7	52.1	52.0	51.9	52.8	52.1	52.2	51.6	51.2	52.3	52.1	51.8	51.8	51.9	74.9	75.4	75.7	75.2	69.8	70.2	75.2	51.9	74.9	74.3	74.3	71.1	74.4	83.7	83.2	82.9	83.1	83.4	83.8	83.8	82.9	83.8	81.6	81.4	83.2	81.1	81.1	53.6	52.8	58.9	77.6	X																																			
48	52.8	52.8	53.2	52.8	53.0	53.9	53.0	52.7	52.8	52.4	53.4	53.0	52.7	52.7	53.0	76.8	77.0	75.4	74.8	70.0	70.5	75.2	53.0	76.8	77.0	77.0	71.7	76.3	99.8	97.8	97.5	97.6	97.9	99.8	99.5	98.7	99.5	81.7	83.1	97.8	82.9	82.9	55.1	56.4	59.1	79.8	83.9	X																																			
49	53.0	53.1	53.4	53.1	53.2	54.1	53.2	52.9	53.0	52.7	53.6	53.2	52.9	52.9	53.2	76.5	76.4	75.7	75.2	70.2	69.5	75.6	53.2	76.5	76.5	76.5	71.9	75.8	98.0	99.6	99.6	99.8	99.8	98.1	97.6	97.1	97.6	81.4	82.5	99.9	82.3	82.3	54.9	56.0	58.7	80.5	83.1	97.9	X																																			
50	52.3	52.2	52.5	52.4	52.3	53.2	52.5	52.7	52.0	51.7	52.8	52.5	52.3	52.3	52.3	75.2	75.7	76.0	75.5	69.8	70.5	75.6	52.3	75.2	74.6	74.6	71.4	74.7	84.0	83.5	83.2	83.4	83.7	84.1	84.1	83.2	84.1	81.8	81.7	83.5	81.4	81.4	54.1	53.2	59.3	77.9	99.8	84.2	83.4	X																																		
51	47.8	47.9	48.0	47.9	48.0	50.8	48.5	48.4	47.1	46.5	48.5	48.0	47.7	47.7	48.0	71.2	71.7	69.8	69.5	66.8	69.5	70.0	48.0	71.2	72.4	72.4	69.0	72.6	79.2	79.6	79.3	79.5	79.8	79.5	79.0	78.8	79.2	77.4	77.6	79.6	77.4	77.4	51.9	52.5	54.3	86.2	79.2	79.2	79.6	79.5	X																																		
52	48.7	48.8	49.2	49.0	48.9	50.1	49.2	49.1	48.6	48.5	49.2	49.2	48.9	48.9	48.9	74.1	74.4	70.9	70.6	65.5	67.0	70.9	48.9	74.1	71.1	71.1	68.8	73.4	78.6	78.6	78.9	78.4	78.7	78.9	78.4	78.2	78.6	75.6	73.7	78.6	73.6	73.7	54.1	54.5	52.1	82.8	76.2	78.6	78.6	76.5	81.5	X																																
53	49.4	49.7	49.6	49.7	49.6	50.1	49.6	50.0	49.1	48.5	50.1	49.6	49.6	49.6	49.6	68.6	68.6	68.8	68.3	61.1	64.2	68.6	49.6	68.6	69.5	69.5	66.6	68.0	73.5	73.4	73.1	73.3	73.6	73.8	73.4	73.0	73.4	73.0	70.8	73.4	70.7	70.6	50.3	51.0	53.4	78.1	74.1	73.8	73.4	74.4	77.3	76.7	X																																
54	52.8	52.6	53.2	52.8	52.8	53.4	53.0	52.7	52.5	52.2	53.2	53.0	52.5	52.5	52.8	75.6	75.6	74.6	74.4	69.3	70.4	74.7	52.8	75.6	75.4	75.4	70.5	74.2	83.3	83.1	82.8	82.9	83.2	83.5	83.2	82.5	83.2	80.8	79.3	83.1	79.1	79.1	56.4	55.8	58.9	78.6	85.2	83.2	82.9	85.5	77.8	76.7	72.9	X																																
55	52.1	52.0	52.5	52.2	52.1	53.2	52.3	52.5	51.8	52.0	52.5	52.3	51.8	51.8	52.1	74.6	74.7	73.7	73.5	68.9	69.9	74.1	52.1	74.6	76.0	76.0	70.7	74.7	80.2	79.8	79.8	79.9	79.9	80.4	80.1	79.4	80.2	81.3	79.1	80.1	79.0	79.0	54.3	53.9	56.6	77.5	83.7	80.1	79.9	83.9	79.6	77.2	72.3	81.9	X																																
56	51.7	51.5	52.1	51.7	51.7	52.3	51.9	51.6	51.3	51.2	52.1	51.9	51.4	51.4	51.7	73.8	73.4	73.4	72.7	69.1	69.9	73.3	51.7	73.8	73.3	73.3	69.5	74.2	82.0	81.7	82.0	81.9	81.9	82.2	81.9	81.3	81.9	79.6	78.8	82.0	78.7	78.7	56.0	53.9	59.1	76.4	83.7	82.2	81.9	83.9	76.4	73.8	71.4	90.8	81.0	X																																
57	53.0	53.1	52.8	53.1	53.0	54.9	53.0	53.1	52.5	52.0	53.2	53.0	52.9	52.9	53.0	77.5	76.7	75.4	75.2	70.7	74.1	75.1	53.0	77.5	77.2	77.2	75.2	77.1	72.5	71.9	71.6	71.6	71.9	72.6	72.4	72.0	72.3	69.8	67.9	71.7	67.9	67.8	56.0	57.9	55.4	70.7	70.0	72.3	71.6	70.4	68.6	69.0	62.5	70.5	71.2	69.0	X																																
58	50.1	50.2	50.5	50.4	50.1	50.8	50.3	50.5	50.1	50.0	50.5	50.3	50.0	50.0	50.1	71.7	72.1	69.1	68.8	65.1	68.1	69.5	50.1	71.7	69.9	69.9	73.8	69.3	70.1	70.0	69.9	70.0	70.0	70.2	70.0	69.5	69.9	68.7	68.4	70.2	67.7	67.6	51.2	51.2	55.6	69.3	69.2	69.9	70.0	69.5	69.5	66.5	64.8	70.2	67.9	70.7	71.2	X																																
59	48.7	48.8	48.9	49.0	48.7	50.5	49.2	49.1	48.4	48.5	48.9	48.9	48.7	48.7	48.7	71.6	71.1	73.1	72.7	63.8	68.8	72.9	48.7	71.6	71.9	71.9	74.9	71.7	68.1	68.3	68.3	68.3	68.3	68.4	68.2	67.6	68.1	68.7	69.5	68.4	69.1	69.1	49.4	50.1	55.6	68.3	69.2	68.1	68.3	69.5	66.8	65.7	63.5	67.2	68.8	68.3	73.3	75.1	X																																
60	42.8	42.7	42.6	42.4	42.8	44.1	43.1	43.0	42.8	42.2	43.3	42.6	42.5	42.5	42.8	56.5	56.9	51.3	51.3	52.8	51.1	51.5	42.8	56.5	52.2	52.2	58.3	56.6	55.4	55.8	55.8	55.6	55.6	55.6	55.6	54.9	55.4	55.1	54.3	55.8	53.9	53.8	46.3	45.8	48.2	54.8	52.6	55.2	55.6	53.1	50.2	50.6	51.1	55.0	52.4	53.3	54.8	52.4	48.6																																

Column1	Column2
1	Georgia_(126)
2	Florida/West_Virginia_(FL16/WV14)
3	Texas/Minnesota/Missouri_(TX_RAC2/RacI/15-7986)
4	Missouri_(RACL)
5	Florida_(FL11)
6	California_(SF2)
7	California_(CC3)
8	California_(SM3)
9	Georgia_(169)
10	Missouri_(MOI)
11	West_Virginia_(WV3)
12	Minnesota_(15-11512)
13	Georgia_(RAC123)
14	Florida_(FL32)
15	Florida_(FL31)
16	Babesia_sp._H1
17	Babesia_sp._Hty-3F
18	California_MAR6
19	California_MAR3
20	Babesia_conradae
21	Babesia_sp._FD-879
22	California_SM1
23	Florida/Ontario_Canada_(FL13/W504-15/W1110-15)
24	Babesia_sp._Rach6_Rachhym_Waterhym
25	Georgia_RAC_008
26	Georgia_RAC122
27	Theileria_youngi
28	Babesia_sp._LPM_chichi
29	Pennsylvania_PAF_12_{lotori}
30	Missouri_RACQ_{maned_wolf}
31	California_SM2_{maned_wolf}
32	California_SON4_{maned_wolf}
33	Idaho_ID2_{maned_wolf}
34	Pennsylvania_PAF_9_{lotori}
35	West_Virginia_WV_10_{lotori}
36	Pennsylvania_PAF_6_{lotori}
37	West_Virginia_WV16_{lotori}
38	Colorado_CO8_{Babesia_sp._new}
39	Pennsylvania_PA1_{Babesia_sp._new}
40	Missouri_RACP_{maned_wolf}
41	Ontario_Canada_W507-15_{Babesia_sp._new}
42	Minnesota_15-7455_{Babesia_sp._new}
43	Babesia_vulpes_MES-2012
44	Babesia_microti_Munich
45	Babesia_rodhaini
46	Babesia_sp._Coco
47	Babesia_gibsoni_HUVIMdr2
48	Babesia_lotori_AJB-2006
49	Babesia_sp._maned_wolf
50	Babesia_gibsoni
51	Babesia_caballi
52	Babesia_bigemina
53	Babesia_bovis
54	Babesia_canis_canis
55	Babesia_canis_rossi
56	Babesia_canis_vogeli
57	Cytauxzoon_felis
58	Theileria_orientalis
59	Theileria_parva
60	Plasmodium_falciparum_WR80