Molecular Characterization of a Babesia Species Identified in a North American Raccoon

Authors: Birkenheuer, Adam J., Whittington, Julia, Neel, Jennifer, Large, Edward, Barger, Anne, et al.

Source: Journal of Wildlife Diseases, 42(2) : 375-380

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-42.2.375

Journal of Wildlife Diseases, 42(2), 2006, pp. 375–380

Molecular Characterization of a *Babesia* Species Identified in a North American Raccoon

Adam J. Birkenheuer,[1,4] Julia Whittington,[2] Jennifer Neel,[1] Edward Large,[1] Anne Barger,[3] Michael G. Levy,[1] and Edward B. Breitschwerdt[1] [1] North Carolina State University, College of Veterinary Medicine, 4700 Hillsborough Street, Raleigh, North Carolina 27606, USA[1]; [2] University of Illinois, College of Veterinary Medicine, Wildlife Medical Clinic, 1008 W. Hazelwood Dr., Urbana, Illinois 61802, USA[1]; [3] University of Illinois, College of Veterinary Medicine, Department of Pathobiology, 2001 S. Lincoln Ave., Urbana, Illinois 61802, USA; [4] Corresponding author (email: ajbirken@ncsu.edu)

ABSTRACT: Piroplasmosis was first described in raccoons (*Procyon lotor*) in 1926, and the official description of a small piroplasm as *Babesia lotori* was done in 1981. *Babesia microti*-like gene sequences have been characterized in raccoons in both North American and Japan. It is well documented that the microscopic appearance of piroplasms does not always accurately predict the genotype and phylogenetic classification. Discrepancies using phenotype to predict genotype have been reported most frequently when evaluating small piroplasms. We amplified and sequenced the full-length 18S rRNA gene from a small piroplasm identified in a raccoon and used this sequence for phylogenetic analyses. Based on these analyses, the organism was placed in the *Babesia* sensu stricto clade, confirming that it is a true *Babesia* sp. This documents that at least two *Babesia* spp. can infect raccoons. The data generated in this study can be used to design molecular diagnostic tests for detection of this *Babesia* sp., which will be useful for epidemiologic and comparative phylogenetic studies. As piroplasmosis has been documented with increased frequency in humans in recent years, the results of this study will aid in the recognition of zoonotic babesiosis.

Key words: Babesia, PCR, phylogenetics, piroplasm, *Procyon lotor*, raccoon, taxonomy.

Piroplasmosis was first reported in raccoons in 1926 (Wenyon and Scott, 1926) and is commonly reported from North American raccoons (*Procyon lotor*), where prevalence can reach 96% (Frerichs and Holbrook, 1970; Anderson et al., 1981; Telford and Forrester, 1991). The nomenclature used to describe piroplasms identified in North American raccoons has been confusing due to similarities with the names proposed for other *Babesia* species. A large piroplasm (>3 μm) identified in a Eurasian raccoon dog (*Nyctereutes procyonoides*) was named *Babesia procyoni*. Likewise, early reports describing a small piroplasm (<3 μm) of North American raccoons referred to this parasite as *B. procyoni* or *B. procyonis* (Frerichs and Holbrook, 1970; Ristic et al., 1977; Anderson et al., 1979). Anderson et al. (1981) differentiated the piroplasms of North American raccoons from the piroplasms of the Eurasian raccoon dogs; they proposed that the name *Babesia lotori* should be used for the small piroplasms of North American raccoons and that *Babesia procyonis* should be used for the large piroplasms of the Eurasian raccoon dog. Adding to the confusion, results from recent molecular studies based on 18S ribosomal ribonucleic acid (rRNA) gene sequences from small piroplasms from raccoons in North America and Japan indicated that sequences were most similar to *Babesia microti*, the most common agent of human babesiosis (Goethert and Telford, 2003; Kawabuchi et al., 2005).

These and other molecular studies demonstrate that parasite morphology and mammalian host species are not always adequate to identify piroplasms accurately. Sequence differences have been reported from morphologically indistinguishable piroplasms (Kjemtrup et al., 2000; Zahler et al., 2000; Birkenheuer et al., 2004). Some small piroplasms, previously believed to be *Babesia* spp., are genetically much more similar to *Theileria* spp. (Mehlhorn and Schein, 1998; Zahler et al., 2000); and the species has undergone a formal reclassification from *Babesia equi* to *Theileria equi* (Mehlhorn and Schein, 1998). There appears to

be at least one other clade of small piroplasms termed the *Babesia microti*-like group that do not group with either the *Babesia* or *Theileria* spp. (Birkenheuer et al., 2004). Others have proposed that there may be as many as five distinct groups within the *Theileria* and *Babesia* genera (Criado-Fornelio et al., 2003). The objective of this study was to genetically characterize a small piroplasm identified in a North American raccoon.

A juvenile male raccoon, approximately 6 months of age, was presented for care after being found nonambulatory along a rural road in Piatt county, Illinois (40.03°N, 88.57°W). Blood was collected via jugular venipuncture, and hematologic evaluation revealed pronounced anemia (18.1%, reference range: 36.8±5.4), hypoproteinemia (5.7 g/dl, reference range: 7.2±0.7), hypoalbuminemia (2.8 g/dl, reference range: 3.4±0.3), elevated blood urea nitrogen (76.1 mg/dl, reference range: 20.0±7.0), hyperphosphatemia (6.5 mg/dl, reference range: 4.7±1.2), hypokalemia (3.0 mEq/L, reference range: 4.3±0.4), hyperglycemia (152 mg/dl, reference range: 60±22), and elevated alanine aminotransferase (874 IU/L, reference range: 121±36). The total white-blood-cell count was within normal reference range (13.6 K/μl, reference range: 9.8±4.1) with the exception of an increased number of monocytes (952 cells, reference range: 333±254) and the presence of immature neutrophils (2% of all cells), suggesting a mild degenerative left shift (Denver, 2003). Microscopic examination of Giemsa-stained blood smears resulted in the identification of *Babesia*-like organisms. Microscopic examination of Romanowsky-stained blood smears resulted in the identification of parasites present within <1% of mature red blood cells (RBC). These organisms were round, oval, amoeboid, or, rarely, piriform in shape, with typically one and rarely two organisms per infected RBC. Based on measurements from 20 organisms, length varied from 1.25 to 4.8 μm and width varied from 0.75 to 1.7 μm; the average width and length were 3.13±0.77 μm and 2.5±0.61 μm, respectively. Organisms had pale-staining cytoplasm and a light-blue outer membrane that varied in thickness from thin and delicate to up to 0.5 μm wide. The nuclei were eccentric and associated with the outer membrane, magenta staining, and round to oval in shape. Size of the nuclei varied from 0.5×0.5 μm up to 1.0×1.6 μm (Fig. 1). A cryopreserved stabilate was prepared using 10% v/v glycerol; however, the viability of this stabilate has not yet been tested.

The raccoon was treated with amoxicillin trihydrate/clavulanate potassium (Clavamox, Pfizer Animal Health, Exton, Pennsylvania, USA) and fendbendazole (Panacur, Intervet Inc., Millsboro, Delaware, USA) initially, followed by clindamycin (Antirobe, Phoenix Scientific, Inc., St. Joseph, Missouri, USA). *Babesia*-like organisms were identified in serial peripheral blood smears intermittently until the time of the raccoon's release (5 mo after presentation). It is unknown if the *Babesia* sp. contributed to clinical disease or was an incidental finding; however, clindamycin has been demonstrated to have efficacy against *Babesia* spp. and this treatment may have contributed to clinical recovery (Wijaya et al., 2000; Wulansari et al., 2003).

DNA isolated from raccoon blood (QIAamp DNA Blood Mini Kit, Qiagen Inc., Valencia, California, USA) was used as a template to amplify the nearly full-length 18S ribosomal DNA. For the polymerase chain reaction (PCR), a Progene Thermocycler (Techne Inc., Princeton, New Jersey, USA) was used, and reactions that utilize a high-fidelity thermostable Tli DNA Polymerase (Promega Corp, Madison, Wisconsin, USA) and primers 5-22F (5′-GTTGATCCTGCCAGTAGT-3′) and 1179-1661R (5′-AACCTTGTTACGACT TCTC-3′) (Integrated DNA Technologies, Coralville, Iowa, USA) were done as previously described (Birkenheuer et al., 2003). After electrophoresis and ethidium

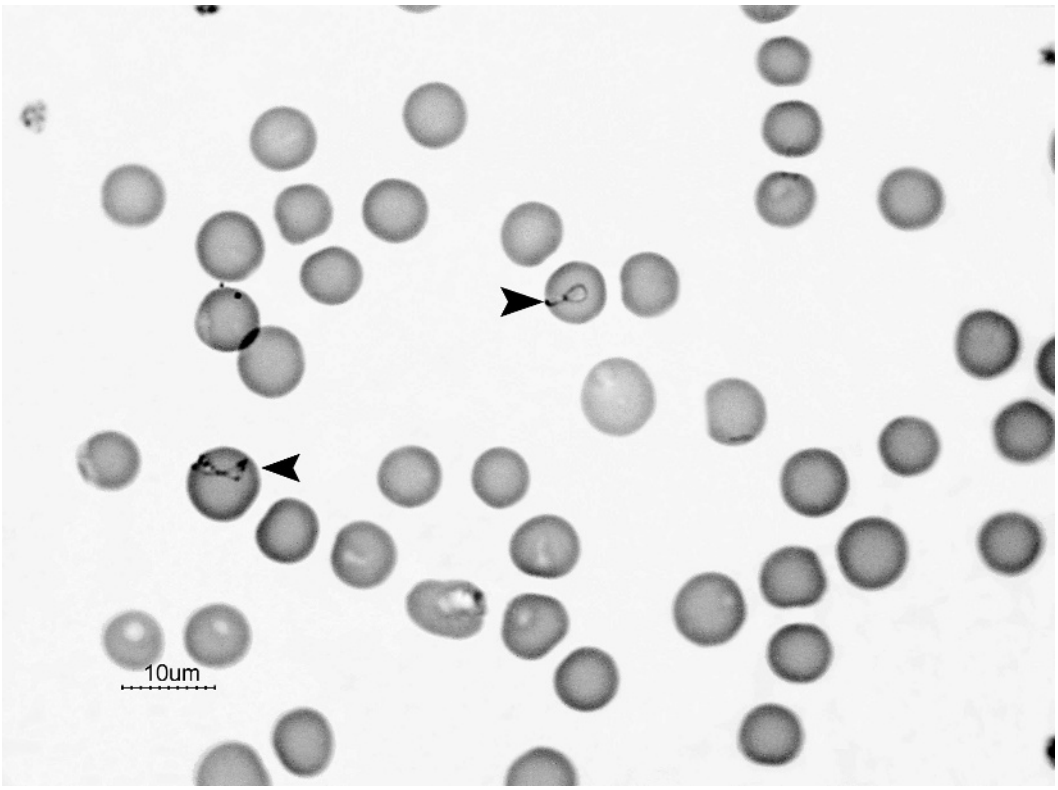

FIGURE 1. Photomicrograph of *Babesia* sp.-infected raccoon red blood cells with single and paired piriform-shaped merozoites (arrowheads). Giemsa-stained thin blood smear 1,000× magnification.

bromide staining, the 1656 base pair PCR product was visualized on an agarose gel with the appropriate DNA size standards (Hyperladder I, Denville Scientific Inc, Metuchen, New Jersey, USA). The PCR product was purified with a commercial kit (QIAquick PCR purification kit, Qiagen Inc.) and submitted for bidirectional sequencing on an ABI 3730 DNA sequencer (Davis Sequencing, Davis, California, USA). The 18S rRNA gene sequence was submitted to GenBank (accession number DQ028958). Comparisons with GenBank sequences were made with the use of the basic local alignment search tool (BLAST) (Altschul et al., 1990), and, based on a nucleotide-nucleotide BLAST search (blastn) with the default settings, the 18S raccoon rRNA sequence was unique. For phylogenetic comparisons, the corresponding 18S sequences of *B. microti*-like species from raccoons, *Babesia canis rossi, B. canis vogeli, B. canis canis, Babesia gibsoni* (Asian genotype), *B. gibsoni* (California genotype), *Theileria annae, Babesia bigemina, Babesia caballi, Babesia odocoilei, B. divergens, Babesia bovis, B. microti, Babesia rodhaini, Theileria equi, Theileria parva, Theileria taurotragi, Theileria annulata, Babesia* sp. RD1, *Babesia* sp. RD61, *Babesia* sp. RD63, *Babesia* sp. MO1, *Babesia* sp. WA1 isolate CA5, *Babesia* sp. WA1 isolate CA6, *Babesia* spp. isolated from ticks in Japan, *Babesia* spp. isolated from a dog, and *Plasmodium falciparum* were included in an alignment. Sequence alignments, excluding the primer sequences, were constructed with the use of Clustal W in the Bioedit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The alignment con-

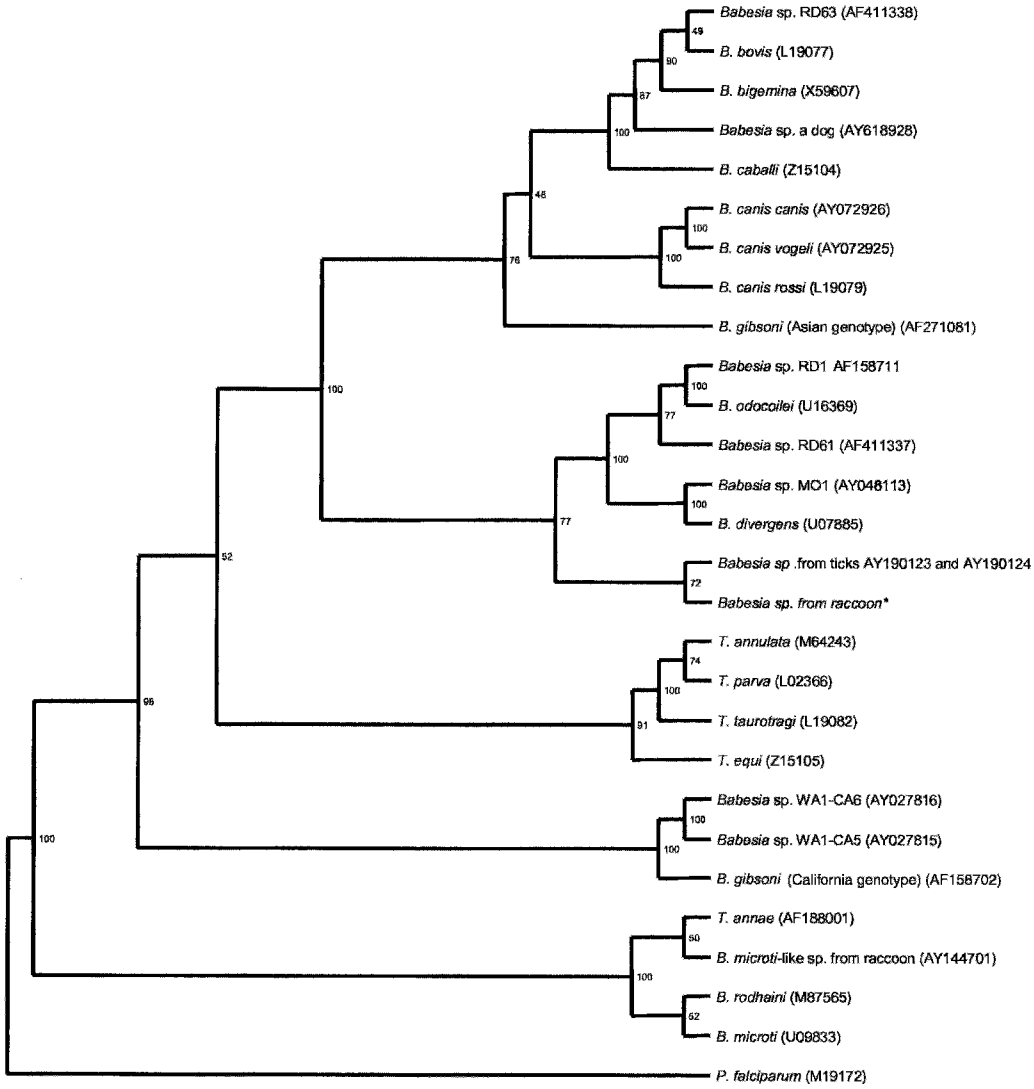

FIGURE 2. Phylogenetic tree inferred from an unedited alignment using parsimony methods demonstrating that *Babesia* species identified in this study is in the *Babesia* sensu stricto clade. The labels next to each node indicate the number of replicates out of 100 in which the group to the right of the node appeared together. The asterisk indicates 18S rRNA gene sequence originating from this study.

sisted of 1,969 nucleotide positions. Cladogram construction and bootstrap analyses were performed with the use of the PHYLIP 3.6 package. Trees were constructed by distance, parsimony, and maximum-likelihood methods with the use of the DNADIST and KITCH, DNAPARS, and DNAML programs, respectively. Bootstrap analyses (1,000 replicates/tree for distance methods and 100 replicates/tree for parsimony and maximum-likelihood methods) were performed with the use of the SEQBOOT and CONSENSE programs. Trees were edited with Microsoft Word.

Our analyses suggest that *Babesia* sp. identified in this raccoon is a true *Babesia* species, as it was placed in the *Babesia* sensu stricto clade with excellent statistical support by all methods; all trees had

similar topology (Fig. 2). Within the *Babesia* sensu stricto clade, this organism formed a monophyletic group with two recently described *Babesia* spp. from ixodid ticks collected from dogs in Japan (Inokuma et al., 2003). Little is known about the two *Babesia* spp. It is not known if these *Babesia* spp. originated from the host species (dog) or had been acquired from a different host prior to attachment to the dogs. No morphologic or infection studies have been performed, so the parasite morphology and natural hosts of these genotypes remain unknown.

This study demonstrates that there are at least two genetically distinct *Babesia* spp. that can infect raccoons. One species appears to be similar to *B. microti*, the most commonly reported agent of human babesiosis in North America, and the other (identified in this study) is a member of the *Babesia* sensu stricto clade. Morphologic analyses alone may not be adequate to accurately identify which *Babesia* sp. is the cause of raccoon babesiosis. Babesiosis of both humans and animals is being diagnosed with increased frequency and the host specificity of *Babesia* spp. does not appear to be as specific as once believed (Homer et al., 2000; Herwaldt et al., 2003; Criado Fornelio et al., 2004). Of the over 100 *Babesia* spp. described to date, genetic data have been reported for less than 20. The data from this study will be helpful in future epidemiologic and molecular phylogenetic studies of the order *Piroplasmida*, and will aid in the recognition of potential zoonotic babesiosis caused by the *Babesia* spp. that infect raccoons.

LITERATURE CITED

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215: 403–410.

Anderson, J. F., L. A. Magnarelli, C. S. Donner, A. Spielman, and J. Piesman. 1979. Canine *Babesia* new to North America. Science 204: 1431–1432.

———,———, and A. J. Sulzer. 1981. Raccoon babesiosis in Connecticut, USA: *Babesia lotori* sp. n. Journal of Parasitology 67: 417–425.

Birkenheuer, A. J., M. G. Levy, and E. B. Breitschwerdt. 2003. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. Journal of Clinical Microbiology 41: 4172–4177.

———, J. Neel, D. Ruslander, M. G. Levy, and E. B. Breitschwerdt. 2004. Detection and molecular characterization of a novel large *Babesia* species in a dog. Veterinary Parasitology 124: 151–160.

Criado-Fornelio, A., A. Martinez-Marcos, A. Buling-Sarana, and J. C. Barba-Carretero. 2003. Molecular studies on *Babesia, Theileria* and *Hepatozoon* in southern Europe. Part II. Phylogenetic analysis and evolutionary history. Veterinary Parasitology 114: 173–194.

———, M. A Gonzalez Del Rio, A. Buling Sarana, and J. C. Barba Carretero. 2004. The "expanding universe" of piroplasms. Veterinary Parasitology 119: 337–345.

Denver, M. 2003. Zoo and wild animal medicine. W. B. Saunders, Philadelphia, pp. 518–519.

Frerichs, W. M., and A. A. Holbrook. 1970. *Babesia* spp. and *Haemobartonella* sp. in wild mammals trapped at the Agricultural Research Center, Beltsville, Maryland. Journal of Parasitology 56: 130.

Goethert, H. K., and S. R. Telford, III. 2003. What is *Babesia microti*? Parasitology 127: 301–309.

Herwaldt, B. L., S. Caccio, F. Herlinzoni, H. Aspock, S. B. Lemenda, P. Piccaluga, G. Martinelli, R. Edelhofer, U. Hollenstein, G. Poletti, S. Pampiglione, K. Loschenberger, S. Tura, and N. J. Pieniazek. 2003. Molecular characterization of a non-*Babesia* divergens organism causing zoonotic babesiosis in Europe. Emerging Infectious Diseases 9: 942–948.

Homer, M. J., I. Aguilar-Delfin, S. R. Telford, III, P. J. Krause, and D. H. Persing. 2000. Babesiosis. Clinical Microbiological Reviews, 13: 451–469.

Inokuma, H., Y. Yoshizaki, Y. Shimada, Y. Sakata, M. Okuda, and T. Onishi. 2003. Epidemiological survey of Babesia species in Japan performed with specimens from ticks collected from dogs and detection of new *Babesia* DNA closely related to *Babesia odocoilei* and *Babesia divergens* DNA. Journal of Clinical Microbiology 41: 3494–3498.

Kawabuchi, T., M. Tsuji, A. Sado, Y. Matoba, M. Asakawa, and C. Ishihara. 2005. *Babesia microti*- like parasites detected in feral raccoons (*Procyon lotor*) captured in Hokkaido. Japanese Journal of Veterinary Medical Science 67: 825–827.

Kjemtrup, A. M., A. A. Kocan, L. Whitworth, J. Meinkoth, A. J. Birkenheuer, J. Cummings, M.

K. Boudreaux, S. L. Stockham, A. Irizarry-Rovira, and P. A. Conrad. 2000. There are at least three genetically distinct small piroplasms from dogs. International Journal of Parasitolology 30: 1501–1505.

Mehlhorn, H., and E. Schein. 1998. Redescription of *Babesia equi* Laveran, 1901 as *Theileria equi* Mehlhorn, Schein 1998. Parasitology Research 84: 467–475.

Ristic, M., G. E. Lewis, Jr., and J. P. Kreier. 1977. *Babesia* in man and wild and laboratory-adapted mammals. *In* Parasitic protozoa, Vol. IV. *Babesia, Theileria, Myxosporida, Microsporida, Bartonellaceae, Anaplasmataceae, Ehrlichia*, and *Pneumocystis*. Academic, New York, pp. 53–76.

Telford, S. R., Jr. and D. J. Forrester. 1991. Hemoparasites of raccoons (*Procyon lotor*) in Florida. Journal of Wildlife Diseases 27: 486–490.

Wenyon, C. M., and H. H. Scott. 1926. Untitled manuscript. Transactions Royal Society Tropical Medicine and Hygiene 20: 6.

Wijaya, A., R. Wulansari, H. Ano, H. Inokuma, and S. Makimura. 2000. Therapeutic effect of clindamycin and tetracycline on *Babesia rodhaini* infection in mouse model. Journal of Veterinary Medical Science 62: 835–839.

Wulansari, R., A. Wijaya, H. Ano, Y. Horii, T. Nasu, S. Yamane, and S. Makimura. 2003. Clindamycin in the treatment of *Babesia gibsoni* infections in dogs. Journal of the American Animal Hospital Association 39: 558–562.

Zahler, M., H. Rinder, E. Zweygarth, T. Fukata, Y. Maede, E. Schein, and R. Gothe. 2000. "*Babesia gibsoni*" of dogs from North America and Asia belong to different species. Parasitology 120 (Pt 4): 365–369.

Received for publication 6 May 2005.