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Source: Journal of Wildlife Diseases, 36(3) : 518-530

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-36.3.518

Journal of Wildlife Diseases, 36(3), 2000, pp. 518–530

ANTIGENIC, PHENOTYPIC AND MOLECULAR CHARACTERIZATION CONFIRMS *BABESIA ODOCOILEI* ISOLATED FROM THREE CERVIDS

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ABSTRACT: *Babesia* isolates from an elk (*Cervus elaphus canadensis*) and a caribou (*Rangifer tarandus caribou*) with fatal infections were compared to *Babesia odocoilei* (Engeling isolate) from white-tailed deer (*Odocoileus virginianus*) by experimental infection, serologic, and small subunit ribosomal RNA (SSU rRNA) gene sequence analysis studies. Both the indirect fluorescent antibody test and immunoprecipitation assays demonstrated antigenic variation among the isolates. Experimental infection studies showed no clinical differences among the isolates. Nucleotide sequence analysis showed that the elk and caribou *Babesia* sp. isolates possessed SSU rRNA genes with identical sequences to that of *B. odocoilei.* A phylogenetic tree constructed from SSU rRNA gene sequences shows that *B. odocoilei* is most closely related to *Babesia divergens,* both of which branch together in the true babesia clade.

Key words: Babesia odocoilei, Cervus elaphus canadensis, Odocoileus virginianus, organism characterization, *Rangifer tarandus caribou,* small subunit ribosomal RNA.

INTRODUCTION

Babesia odocoilei, a babesia of white-tailed deer (*Odocoileus virginianus*), is widely distributed in eastern Texas and Oklahoma (USA), and is assumed to be still present in the southeastern United States where it was originally described (Emerson and Wright, 1968; 1970; Perry et al., 1985; Robinson et al., 1968; Waldrup, 1991; Waldrup et al., 1989). Dual *Theileria* and *Babesia* spp. infections have been described in white-tailed deer, and it has been suggested that the latter causes significant mortality among free-ranging animals (Robinson et al., 1968). In stressful situations hemolytic anemia, anorexia, and sometimes death have been reported (Gray et al., 1991; Waldrup et al., 1989). However, others have suggested that infection exists without compromising the host, which has been supported by results from experimental infections in spleen intact deer (Perry et al., 1985; Waldrup, 1991). The susceptibility of other cervine hosts to *B. odocoilei* has not been determined in either free-ranging or managed environments.

The first reports of naturally acquired acute fatal babesiosis in cervidae under management were described in a caribou (*Rangifer tarandus caribou*) in the Minnesota Zoological Garden (Apple Valley, Minnesota, USA) and in a North American elk (*Cervus elaphus canadensis*) in a farmed herd near Del Rio (Texas, USA) (Holman et al., 1994a, b; Petrini et al., 1995). In both cases, the *Babesia* sp. isolates were small parasites which tended to be located intracellularly adjacent to the host erythrocyte margin, with morphology very similar to that of *B. odocoilei.* This morphological phenotype is also shared by the European parasites *Babesia capreoli* of roe deer (*Capreolus capreolus*) and red deer (*Cervus elaphus elaphus*) and *Babesia divergens* of cattle, which also has been shown to infect cervidae under experimental conditions (Enigk and Friedhoff, 1962a, b; 1963; Nilsson et al., 1965; Gray et al., 1990).

Traditionally, *Babesia* species have been

delineated based on the host of origin, clinical course of infection, and the tick vector. Serologic methods, such as the immunofluorescent antibody (IFA) test, demonstrating specific reactions are also useful tools (Waldrup et al., 1992; Thomford et al., 1993; Goff et al., 1993). Ultimately, however, the best measure of identity or relatedness of organisms lies at the molecular level. The small subunit ribosomal RNA (SSU rRNA) gene is an excellent candidate for molecular taxonomic studies because it codes for a macromolecule that is functionally equivalent in all species, and the consensus sequences involved in the secondary structure motif allow alignment of sequences from diverse organisms.

The main objective of the present study was to clarify the relationships among *B. odocoilei* and two cervine *Babesia* spp. isolates which shared similar morphological characteristics, but differed in their mammalian host of origin, geographic source, and apparent pathogenicity. Infection studies in red deer provided information on host specificity, clinical profiles, and specific antiserum for serologic analyses, which included immunoprecipitation and immunofluorescent antibody (IFA) assays. The infections in these animals were followed by vitro cultivation techniques for *Babesia* spp. Also using cultivation techniques, we investigated the role of white-tailed deer resident at the Minnesota Zoological Garden as possible reservoirs of infection. Lastly, the construction of a phylogenetic tree from SSU rRNA gene sequences of the *Babesia* spp. isolated from the caribou and elk, *B. odocoilei, B. divergens,* and other hemoprotozoan parasites showed taxonomic relationships both among these cervine isolates and to other related hemoprotozoan parasites.

MATERIALS AND METHODS

Babesia spp. isolates

The Brushy Creek isolate of *Babesia odocoilei* (*B. odocoilei*-B) was obtained from a naturally infected deer on the Brushy Creek Experimental Ranch located in the Gulf Slope area of Texas (95°25′W, 31°00′N). The isolate was established in vitro using previously described techniques (Holman et al., 1988). The Engeling isolate of *B. odocoilei* (*B. odocoilei*-E) was originally acquired during a study of deer diseases (Holman et al., 1988). The isolate was obtained as an infected blood sample drawn from a 1.5-yr-old male white-tailed deer that had been killed by a hunter at the Gus Engeling Wildlife Management Area (Anderson County, Texas; 96°26′W, 32°55′N). Both isolates were classified as *B. odocoilei* based on host of origin and morphological characteristics observed on Giemsa-stained blood films as previously described for *B. odocoilei* of white-tailed deer (Spindler et al, 1958; Emerson and Wright, 1968; Perry et al., 1985).

The caribou and elk *Babesia* spp. isolates originated from animals that died during infection with high circulating parasitemias and have been described (Holman et al., 1994a, b)

The *B. divergens* strain originated from an infected Hereford crossbred heifer in County Wicklow, Ireland (Purnell et al., 1976). *Babesia divergens*-infected gerbil (*Meriones unguiculatus*) blood was kindly provided by J. S. Gray (Department of Agricultural Zoology and Genetics, University College-Dublin, Belfield, Dublin, Republic of Ireland). The parasites were adapted to culture in bovine erythrocytes using methods previously described for adapting *Babesia bovis* to culture in exogenous erythrocytes (Holman et al., 1993b).

Experimental infection studies and antiserum production

The caribou and elk *Babesia* spp. isolates and *B. odocoilei*-E were compared in vivo by experimental infection of yearling male red deer (*Cervus elaphus elaphus*), to which the North American elk (*Cervus elaphus canadensis*) is a closely related subspecies. The animals were housed in indoor facilities at the Texas A&M University Research Park (College Station, Texas, USA).

The inocula were cryopreserved parasites cultured in donor RBC obtained from a yearling male red deer (R92). Prior to use for parasite cultures, an aliquot of donor R92 RBC was subjected to culture conditions to ensure that the animal was *Babesia* sp.-free. The animal also was serologically negative for *B. odocoilei* by the indirect immunofluorescence assay.

Inocula of 2×10^9 infected erythrocytes (iRBC) were prepared from cultures of the caribou isolate in the 14th passage, the elk isolate in the 15th passage, and from *B. odocoilei*-E in

excess of passage 50, calculated as follows: The percent iRBC in each culture was determined from microscopic examination of at least 1,000 cells in Giemsa-stained blood cell smears. The total number of RBCs in each culture was determined by counting aliquots from each culture in a hemacytometer. The appropriate volume of each culture to yield 2×10^9 iRBC was then centrifuged for 10 min at $500 \times g$ to pellet the erythrocytes. The supernatants were removed and the cell pellets resuspended to a total volume of 2 ml with 20% w/v PVP (polyvinylpyrrolidone 40; Sigma Chemical Co., St. Louis, Missouri, USA) in PSGG (Puck's saline glucose with 10% extra glucose w/v added) and stored in liquid nitrogen until use. At present there is no known method for synchronizing in vitro cultures of *Babesia* spp. Since the different isolates grew at different rates in vitro, cryopreserved parasites were used so that simultaneous delivery of equivalent doses of the three isolates to their respective recipients could be achieved.

Prior to inoculation, blood was collected in the anticoagulant ethylenediaminetetraacetic acid (EDTA) by jugular venipuncture from each red deer for Giemsa-stained smears, erythrocyte cultures to ensure that the red deer were free of hemoparasites (or other adventitious organisms), and for baseline packed cell volumes (PCV). Serum samples were also obtained and stored at −70 C until tested for antibody activity in the indirect immunofluorescence assay (IFA).

At inoculation, the cryovials of each *Babesia* sp. isolate were placed on ice until needed. At the animal holding facilities, the samples were rapidly thawed in a 37 C waterbath. Each animal received 2 ml containing 2×10^9 infected erythrocytes intramuscularly. Since our work with *B. bovis* has demonstrated no difference in the subsequent infection/disease whether intramuscular, subcutaneous, or intravenous inoculation is used (unpublished results), we chose the intramuscular route to ensure that the full dose was delivered to that site in all animals. Red deer R87 received the caribou isolate, R89 received the elk isolate, and R92 received *B. odocoilei*-E. Blood samples were drawn beginning on day 6 after inoculation, was continued daily, with a few exceptions, through day 16, and was subsequently performed on days 19, 21, 23, 28, 34, 42, and 49. Giemsa stained smears and serum samples were prepared at each blood drawing; and from day 9 onwards erythrocytes were prepared for culture following previously described procedures for initiating cultures from blood with a low parasitemia (Holman et al., 1993a). Again 8 mo after inoculation, blood samples were drawn for culture, serologic evaluation, and Giemsa-stained blood film examination.

White-tailed deer as a reservoir of *Babesia odocoilei* infection

Blood was obtained from three free-ranging white-tailed deer, including an 8-mo-old female fawn, a 4-yr-old male, and a seven-point male, in residence at the Minnesota Zoological Garden. The blood was collected into EDTA and immediately shipped overnight to Texas A&M University. Upon arrival, the blood was washed three times by centrifugation in Dulbecco's phosphate buffered saline (DPBS), pH 7.4 (GIBCO, Grand Island, New York, USA) containing 15 mM EDTA as previously described (Holman et al., 1994b). The culture medium consisted of H1-1 supplemented with 2 mm l-glutamine and 20% white-tailed deer serum (pooled sera from three deer previously shown to support the growth of *B. odocoilei* in vitro). Erythrocyte cultures from each animal were initiated by drawing 0.2 ml packed washed erythrocytes into a 2.0 ml pipet containing 1.8 ml medium. The 2 ml were dispensed into one well, mixed, and 1 ml transferred to the adjacent well, resulting in two 1-ml cultures for each isolate. The cultures were incubated at 37 C in a humidified modular incubator unit perfused with 2% oxygen, 5% carbon dioxide and 93% nitrogen as previously described (Holman et al., 1994b). Each day 0.7 ml medium overlying the cell layer was removed, RBC thin smears made for Giemsa staining, and 0.7 ml new medium added back to the wells.

Culture of the *Babesia* isolates in white-tailed deer erythrocytes

Babesia odocoilei-E and the caribou and elk *Babesia* spp. isolates were cultured in white-tailed deer erythrocytes to produce antigen for the indirect fluorescent antibody (IFA) test and for metabolic labelling described below. Two donor white-tailed deer housed at the Texas A&M University Research Park provided uninfected donor erythrocytes for the cultures. The deer were determined to be free of hemoparasites by culture, Giemsa-stained blood smears, and IFA tests prior to use. The white-tailed deer erythrocyte suspensions were prepared for culture and the *B. odocoilei* and the caribou and elk *Babesia* spp. reisolated from the inoculated red deer subcultured into white-tailed deer erythrocytes as previously described (Holman et al., 1994b). The isolates were subcultured in white-tailed deer erythrocytes at least five passages before use to ensure dilution elimination of the original host red deer erythrocytes (Holman et al., 1993b).

Indirect fluorescent antibody test

Standard protocols were followed for the IFA test using fluorescein-labeled Protein-G (Sigma) as the conjugate (Goff et al., 1993). Antigen slides were prepared from *B. odocoilei*-E and the caribou and elk *Babesia* isolates cultured in white-tailed deer erythrocytes. Eight weeks after inoculation specific antiserum to each isolate was obtained from the experimentally infected red deer: R87, caribou *Babesia* isolate; R89, elk *Babesia* isolate; R92, *B. odocoilei*-E. The antisera were tested in serial two-fold dilutions beginning at a 1:40 in phosphate buffered saline, pH 7.2. All tests were observed under 1,000× magnification on an American Optical model 120 epifluorescent microscope (American Optical, Scientific Instrument Division, Buffalo, New York, USA).

Immunoprecipitation analysis

Babesia odocoilei-E and the caribou and elk *Babesia* spp. reisolated from the inoculated red deer were cultured in white-tailed deer erythrocytes. The parasites were metabolically labeled by ^{35}S-methionine uptake when the parasitemia exceeded 10% as follows. The medium overlying the erythrocytes was removed and replaced with methionine free-MEM containing 10% FBS and 125 μCi [^{35}S]-methionine/ml. Following an overnight incubation, the erythrocytes were washed three times by centrifugation in DPBS as previously described (Holman et al., 1994b), and the sample prepared for immunoprecipitation by a previously described method (Barbet et al., 1983). [^{35}S]-labeled proteins from each isolate were each reacted separately with 10 μl anti-caribou *Babesia* sp. red deer serum (from R87), 10 μl anti-elk *Babesia* sp. red deer serum (from R89), 10 μl anti- *B. odocoilei* red deer serum (from R92), and 10 μl normal red deer serum (preimmune serum from R89). The resulting immunocomplexes were precipitated with Protein G bound to Sepharose beads (Barbet et al., 1983). The immunoprecipitates were solubilized by boiling in SDS-PAGE sample buffer for 3 min and then were electrophoresed in a 7.5% to 17.5% SDS-PAGE gel with a 4% stacking gel. The SDS-PAGE separated antigen-antibody complexes were visualized by autoradiography as previously described (Barbet et al., 1983).

Babesia spp. SSU rRNA gene amplification

Genomic DNA was extracted from cultured parasites and white-tailed deer leukocytes using standard phenol-chloroform techniques (Sambrook et al., 1989). *Babesia divergens* was cultured in bovine RBC, and *B. odocoilei*-E, *B. odocoilei*-B, and the *Babesia* spp. isolates from the caribou and the elk were cultured in white-tailed deer erythrocytes. *Babesia* spp. SSU rRNA genes were amplified from genomic DNA using primers A and B as previously described (Allsopp et al., 1989; Sogin et al., 1990), except that the extension step was progressively increased by 30 sec during each cycle in a Perkin-Elmer Cetus DNA thermal cycler (Hoffman-LaRoche Corporation, Norwalk, Connecticut, USA). White-tailed deer leukocyte genomic DNA was subjected to the same PCR conditions to confirm the specificity of these conditions to amplify only piroplasm SSU rDNA. The resulting product of approximately 1,800 bp was either directly sequenced after gel purification, or cloned into pCR-Script™ following manufacturer's instructions (Stratagene Cloning Systems, La Jolla, California, USA) to facilitate sequence analysis of the 3′ and 5′ ends. The phagemid templates were also used to confirm occasional sequence regions where sequencing directly from PCR gave imprecise data.

Sequencing reactions were labeled with ^{35}S-dATP (Amersham Life Sciences, Arlington Heights, Illinois, USA) by linear amplification using Exo$^-$ *P*fu DNA polymerase according to manufacturer's instructions (Cyclist Exo$^-$ *P*fu DNA Sequencing Kit; Stratagene Cloning Systems) using 100–200 ng template DNA and 5 ng primer. The two series of primers used to sequence the forward and reverse strands are shown in Table 2 (Elwood et al., 1985; Allsopp et al., 1994). Primers complementary to the T7 and T3 promotor regions of the pCR-Script™ vector were also used (Stratagene Cloning Systems). After amplification, the samples were held at 4 C until subjected to 3′ random nucleotide tailing to alleviate strong stops. Tailing was accomplished by incubation with terminal deoxynucleotidyl transferase (United States Biochemicals Corporation, Cleveland, Ohio, USA) according to manufacturer's instructions. At incubation completion 5 μl cold stop solution (Cyclist Exo$^-$ *P*fu DNA Sequencing Kit; Stratagene Cloning Systems) were added to each tube and the tubes were stored at 4–10 C until used. Samples were denatured at 95 C for 3 min, quenched in wet ice, and loaded in 2–4 μl aliquots on a 7 M urea, 6% polyacrylamide gel in a Sequi-Gen Sequencing System apparatus (Bio-Rad Laboratories, Hercules, California, USA). After electrophoresis the gel was dried at 80 C under vacuum, autoradiographed, and the sequences visually read.

Sequence analysis

Complete SSU rRNA gene sequences for the organisms given in Table 1 were retrieved as an

TABLE 1. List of hemoprotozoan species and the outgroup protozoan, *Oxytricha nova*, from which the SSU rRNA gene sequences used for phylogenetic tree inference originated. All sequences were acquired as an alignment from the Ribosomal Database Project database (Larsen et al., 1993), except the *Babesia divergens* and *Babesia odocoilei* SSU rRNA sequences, which were added to the alignment and manually adjusted to conform to it using the Genetic Data Environment program (GDE 2.2; Smith, 1992) sequence editor. The GenBank™ accession number and contributors of each SSU rRNA gene sequence are given.

Species	GenBank number	Source
Babesia bigemina	X59604	Reddy et al., 1991
Babesia bovis 2	L19077	Allsopp et al., 1994
Babesia caballi	Z15104	Allsopp et al., 1994
Babesia canis	L19079	Allsopp et al., 1994
Babesia divergens	U16370	Holman, 1994
Babesia equi	Z15105	Allsopp et al., 1994
Babesia microti	U09833	Allsopp et al., 1994
Babesia odocoilei	U16369	Holman, 1994
Babesia rodhaini	M87565	Ellis et al., 1992
Babesia rodhaini 2		M. T. E. P. Allsopp, unpubl. data
Babesia sp.	U09834	Allsopp, 1994
Oxytricha nova	M14601	Elwood et al., 1989
Theileria annulata	M64243	Gajadhar et al., 1991
Theileria buffeli	Z15106	Allsopp et al., 1994
Theileria parva	L02366	Allsopp et al., 1993
Theileria taurotragi	L19082	Allsopp et al., 1994
Theileria sp.	L19081	Allsopp et al., 1994

alignment from the Ribosomal Database Project (RDP) database (Larsen et al., 1993), except *B. divergens* and the cervine *Babesia* sp. (*B. odocoilei*) sequences acquired during this study, which were added to the alignment and manually adjusted to conform to it using the edit menu of the Genetic Data Environment program (GDE 2.2; Smith, 1992) sequence editor. Members of the Theileriidae and Babesiidae were included to provide a framework of ancestry for the tree topology and to provide an adequate number of sequences to facilitate

TABLE 2. Nucleotide sequences, gene positions, and optimum annealing temperatures for the primers used to amplify and sequence SSU rRNA genes from *Babesia* spp. DNA.

Primer	Position[a]	Primer nucleotide sequence 5′ → 3′	Annealing temperature
A	5′ forward	ccgaattcgtcgacAACCTGGTTGATCCTGCCAGT[a]	60 C
B	5′ reverse	cccgggatccaagcttGATCCTTCTGCAGGTTCACCTAC[a]	60 C
300F[b]	375	AGGGTTCGATTCCGGAG	60 C
528F	578	CGGTAATTCCAGCTCC	50 C
760F	979	ATCAAGAACGAAAGT	50 C
1055F	1282	GGTGGTGCATGGCCG	55 C
1200F	1462	CAGGTCTGTGATGCT	55 C
300R	401	TCAGGCTCCCTCTCCGG	50 C
536RM	582	AATTACCGCGGCTGCTG	60 C
690R	923	AGAATTTCACCTCTG	45 C
1055RM	1298	AACGGCCATGCACCAC	60 C
1200R	1477	GGGCATCACAGACCTG	55 C
T3[c]		AATTAACCCTCACTAAAGGG	50 C
T7[d]		GTAATACGACTCACTATAGGGC	50 C

[a] Polylinker additions are indicated by the lower case letter sequences.
[b] Internal primer names are as designated by Elwood et al. (1985). Forward strand synthesis primers are designated F; reverse strand primers designated R. Slight modification for use with piroplasm sequences is indicated by "M" (Allsopp et al., 1994).
[c] Primer complementary to the T3 promotor region in pCR-Script™ phagemid sequence for forward strand sequencing.
[d] Primer complementary to the T7 promotor region in pCR-Script™ phagemid sequence for reverse strand sequencing.

alignment through the variable regions. The SSU rRNA gene sequence for *Oxytricha nova* served as the outgroup for the phylogenetic analysis because ciliates are considered one of the closest free-living relatives of the Apicomplexa (Gajadhar et al., 1991). Table 1 lists the species of origin, reference, and GenBank™ accession number for all sequences used.

Phylogenetic trees were inferred from the aligned sequences using four different algorhythms in the PHYLIP Version 3.5 phylogeny inference package (Felsenstein, 1993), including the NEIGHBOR joining distance, FITCH distance, fastDNAml maximum likelihood and DNAPARS parsimony methods. The aligned sequences were exported from GDE 2.2 using the PHYLIP format and the exported alignment was used as the input data for fastDNAml and DNAPARS. A genetic distance matrix was calculated from the PHYLIP-formatted alignment using the DNADIST program (Kimura two-parameter substitution model) (PHYLIP Version 3.5), which computed the genetic distance matrix based on the fraction of sites which differed between each pair of sequences for all the species included in the analysis. The resulting distance matrix was then used as the input for the NEIGHBOR and FITCH programs. The options under which the programs were run are given in Table 3. Majority-rule consensus trees were computed for NEIGHBOR, FITCH, and DNAPARS using the SEQBOOT and CONSENSE programs of the PHYLIP Version 3.5 phylogeny package. In each case, the SEQBOOT parameters included a random number seed of 101, with 100 resamplings.

RESULTS

Red deer inoculated with the three *Babesia* sp. isolates did not develop clinical signs as a result of infection, except for a slight drop in PCV between days 12 and 14 after inoculation (Fig. 1). Giemsa-stained blood smears were consistently negative for the presence of *Babesia* organisms. However, *Babesia* sp. parasites were recovered from the inoculated animals by culturing blood samples from day 9 onwards after inoculation (Table 4). The three isolates were recovered from their respective hosts through day 49. At day 77, the caribou *Babesia* sp. isolate and the elk *Babesia* sp. isolate were cultured from R87 and R89, respectively, but no *B. odocoilei*-E parasites were cultured from R92 at this

TABLE 3. Option settings used in the DNADIST, FITCH, NEIGHBOR, DNAPARS, and fastDNAml algorhythms for phylogenetic tree inference. Parameters were set in response to specific queries in the PHYLIP Version 3.5 phylogeny inference package programs (Felsenstein, 1993).

DNADIST	FITCH	NEIGHBOR	NDAPARS	fastNDAml
Distance-Kimura	Search for best tree	Neighbor joining tree	Search for best tree	Search for best tree
	Outgroup root-*Oxytricha nova*	Outgroup root-*Oxytricha nova*	Outgroup root-*Oxytricha nova*	Outgroup root-*Oxytricha nova*
One category of substitution rates	No/no-Upper/lower-triangular data matrix	No/no-Upper/lower-triangular data matrix	Ordinary parsimony	One category of substitution rates
Square distance matrix	Use input order of species	Use input order of species	Use input order of species	Use input order of species
	No subreplicates	No subreplicates		Empirical base frequencies
	Global rearrangements			Global rearrangements
1.5 Transition/transversion ratio		1.5 Transition/transversion ratio		1.5 Transition/transversion ratio
	Power—2.00000			

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Bdv  AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATGTCT   60 Bdv
Bodo AACCTGGTTG ATCCTGCCAG TAGTCATATG CTTGTCTTAA AGATTAAGCC ATGCATTGCT   60 Bod

Bdv  AAGTACAAAC TTTTTACGGT GAAACTGCGA ATGGCTCATT ACAACAGTTA TAGTTTCTTT  120 Bdv
Bod  AAGTACAAAC TTTTTACGGT GAAACTGCGA ATGGCTCATT ACAACAGTTA TAGTTTCTTT  120 Bod

Bdv  GGTATTCGTT TTCCATGGAT AACCGTGCTA ATTGTAGGGC TAATACAAGT TCGAGGCCTT  180 Bdv
Bod  GGTATTCGTT TTCCATGGAT AACCGTGCTA ATTGTAGGGC TAATACAAGT TCGAGGCCTT  180 Bod

Bdv  TT-GGCGGCG TTTATTAGTT CTAAAACCAT CCCTTTTGGT TTTCGGTGAT TCATAATAAA  239 Bdv
Bod  TTTGGCGGCG TTTATTAGTT CTAAA-CCAT CCGTTTTGGT TTTCGGTGAT TCATAATAAA  239 Bod

Bdv  CTTGCGAATC GCAATTTTTT GCGATGGACC ATTCAAGTTT CTGACCCATC AGCTTGACGG  299 Bdv
Bod  CTCGCGAATC GCAATTTATT GCGATGGACC ATTCAAGTTT CTGACCCATC AGCTTGACGG  299 Bod

Bdv  TAGGGTATTG GCCTACCGAG GCAGCAACGG GTAACGGGGA ATTAGGGTTC GATTCCGGAG  359 Bdv
Bod  TAGGGTATTG GCCTACCGAG GCAGCAACGG GTAACGGGGA ATTAGGGTTC GATTCCGGAG  359 Bod

Bdv  AGGGAGCCTG AGAAACGGCT ACCACATCCA AGGAAGGCAG CAGGCGCGCA AATTACCCAA  419 Bdv
Bod  AGGGAGCCTG AGAAACGGCT ACCACATCCA AGGAAGGCAG CAGGCGCGCA AATTACCCAA  419 Bod

Bdv  TCCTGACACA GGGAGGTAGT GACAAGAAAT AACAATACAG GGCAATTGTC TTGTAATTGG  479 Bdv
Bod  TCCTGACACA GGGAGGTAGT GACAAGAAAT AACAATACAG GGCAATTGTC TTGTAATTGG  479 Bod

Bdv  AATGATGGTG ACCTAAACCC TCACCAGAGT AACAATTGGA GGGCAAGTCT GGTGCCAGCA  539 Bdv
Bod  AATGATGGTG ACCTAAACCC TCACCAGAGT AACAATTGGA GGGCAAGTCT GGTGCCAGCA  539 Bod

Bdv  GCCGCGGTAA TTCCAGCTCC AATAGCGTAT ATTAAACTTG TTGCAGTTAA AAAGCTCGTA  599 Bdv
Bod  GCCGCGGTAA TTCCAGCTCC AATAGCGTAT ATTAAACTTG TTGCAGTTAA AAAGCTCGTA  599 Bod

Bdv  GTTGAATTTT TGCGTGGTGT TAATATTGAC TAATGTCGAG ATTGCACTTC CGCTTTTGGG  659 Bdv
Bod  GTTGAATTTC TGCGTCACCG TATT-TTGAC TTTTGTCGAC TGTCGGTTTC CGCTTTTGGG  658 Bod

Bdv  ATTTATCCCT TTTTACTTTG AGAAAATTAG AGTGTTTCAA GCAGACTTTT GTCTTGAATA  719 Bdv
Bod  ATTTATCCCT TTTTACTTTG AGAAAATTAG AGTGTTTCAA GCAGACTTTT GTCTTGAATA  718 Bod

Bdv  CTTCAGCATG GAATAATAGA GTAGGACTTT GGTTCTATTT TGTTGGTTTG TGAACCTTAG  779 Bdv
Bod  CTTCAGCATG GAATAATAGA GTAGGACTTT GGTTCTATTT TGTTGGTTTG TGAACCTTAG  778 Bod

Bdv  TAATGGTTAA TAGGAACGGT TGGGGGCATT CGTATTTAAC TGTCAGAGGT GAAATTCTTA  839 Bdv
Bod  TAATGGTTAA TAGGAACGGT TGGGGGCATT CGTATTTAAC TGTCAGAGGT GAAATTCTTA  838 Bod

Bdv  GATTTGTTAA AGACGAACTA CTGCGAAAGC ATTTGCCAAG GACGTTTTCA TTAATCAAGA  899 Bdv
Bod  GATTTGTTAA AGACGAACTA CTGCGAAAGC ATTTGCCAAG GACGTTTTCA TTAATCAAGA  898 Bod

Bdv  ACGAAAGTTA GGGGATCGAA GACGATCAGA TACCGTCGTA GTCCTAACCA TAAACTATGC  959 Bdv
Bod  ACGAAAGTTA GGGGATCGAA GACGATCAGA TACCGTCGTA GTCCTAACCA TAAACTATGC  958 Bod

Bdv  CGACTAGGGA TTGGAGGTCG TCATTTTTCC GACTCCTTCA GCACCTTGAG AGAAATCAAA 1019 Bdv
Bod  CGACTAGGGA TTGGAGGTCG TCATTTTTCC GACTCCTTCA GCACCTTGAG AGAAATCAAA 1018 Bod

Bdv  GTCTTTGGGT TCTGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA TTGACGGAAG 1079 Bdv
Bod  GTCTTTGGGT TCTGGGGGGA GTATGGTCGC AAGGCTGAAA CTTAAAGGAA TTGACGGAAG 1078 Bod

Bdv  GGCACCACCA GGCGTGGAGC CTGCGGCTTA ATTTGACTCA ACACGGGGAA ACTCACCAGG 1139 Bdv
Bod  GGCACCACCA GGCGTGGAGC CTGCGGCTTA ATTTGACTCA ACACGGGGAA ACTCACCAGG 1138 Bod

Bdv  TCCAGACAAT GTTAGGATTG ACAGATTGAT AGCTCTTTCT TGATTCTTTG GGTGGTGGTG 1199 Bdv
Bod  TCCAGACAAT GTTAGGATTG ACAGATTGAT AGCTCTTTCT TGATTCTTTG GGTGGTGGTG 1198 Bod

Bdv  CATGGCCGTT CTTAGTTGGT GGAGTGATTT GTCTGGTTAA TTCCGTTAAC GAACGAGACC 1259 Bdv
Bod  CATGGCCGTT CTTAGTTGGT GGAGTGATTT GTCTGGTTAA TTCCGTTAAC GAACGAGACC 1258 Bod

Bdv  TTAACCTGCT AACTAGTGTC CGTAAAAAGG TTCGTCCGTT ACGGTTTGCT TCTTAGAGGG 1319 Bdv
Bod  TTAACCTGCT AACTAGTGCC CGTGAGAAGG TTCGTCCGTT ACGGGTTGCT TCTTAGAGGG 1318 Bod

Bdv  ACTTTGCGGC TCTAAGCCGC AAGGAAGTTT AAGGCAATAA CAGGTCTGTG ATGCCCTTAG 1379 Bdv
Bod  ACTTTGCGGC TCTAAGCCGC AAGGAAGTTT AAGGCAATAA CAGGTCTGTG ATGCCCTTAG 1378 Bod

Bdv  ATGTCCTGGG CTGCACGCGC GCTACACTGA TGCATTCATC GAGTTTTATC CCTTCCCGAA 1439 Bdv
Bod  ATGTCCTGGG CTGCACGCGC GCTACACTGA TGCATTCATC GAGTTTTATC CCTGCCCGAA 1438 Bod

Bdv  AGGGCTGGGT AATCTTTAGT ATGCATCGTG ACGGGGATTG ATTTTTGCAA TTCTAAATCA 1499 Bdv
Bod  AGGGCTGGGT AATCTTTAGT ATGCATCGTG ACGGGGATTG ATTTTTGCAA TTCTAAATCA 1498 Bod

Bdv  TGAACGAGGA ATGCCTAGTA TGCGCAAGTC ATCAGCTTGT GCAGATTACG TCCCTGCCCT 1559 Bdv
Bod  TGAACGAGGA ATGCCTAGTA TGCGCAAGTC ATCAGCTTGA GCAGATTACG TCCCTGCCCT 1558 Bod

Bdv  TTGTACACAC CGCCCGTCGC TCCTACCGAT CGAGTGATCC GGTGAATTAT TCGGACCGTG 1619 Bdv
Bod  TTGTACACAC CGCCCGTCGC TCCTACCGAT CGAGTGATCC GGTGAATTAT TCGGACCGTG 1618 Bod

Bdv  GCCTTTCCGA TTCGTCGGCT TGGCCTAGGG AAGTCTTGTG AACCTTATCA CTTAAAGGAA 1679 Bdv
Bod  GCTTTTCCGA TTCGTCGGTT TTGCCTAGGG AAGTCTCGTG AACCTTATCA CTTAAAGGAA 1678 Bod

Bdv  GGAGAAGTCG TAACAAGGTT TCCGTAGGTG AACCTGCAGA AGGATC               1725 Bdv
Bod  GGAGAAGTCG TAACAAGGTT TCCGTAGGTG AACCTGCAGA AGGATC               1724 Bod
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FIGURE 1. Alignment of the nucleotide sequences obtained from *Babesia divergens* and *Babesia odocoilei* SSU rRNA genes. Differences between the two sequences are indicated in bold type and the position underlined. Identical SSU rRNA gene sequences were found in *B. odocoilei*-E, *B. odocoilei*-B, and in the elk *Babesia* sp. and the caribou *Babesia* sp. and are represented as a single *B. odocoilei* sequence.

TABLE 4. Results of *Babesia* sp. erythrocyte cultures initiated from the red deer inoculated with the various isolates. The parasites were detected by microscopic examination of Giemsa-stained erythrocyte films.

Day culture initiated after inoculation	Days to parasite detection		
	Caribou isolate	Elk isolate	*Babesia odocoilei*-E
9	5	4	4
11	3	4	4
12	ND[a]	4	ND
13	4	3	3
14	4	5	4
15	4	5	4
16	4	5	4
19	ND	5	10
21	4	4	4
23	7	6	3
28	5	3	5
34	6	6	4
42	6	4	9
49	6	4	9
77	7	6	NP[b]

[a] ND = not done.
[b] NP = no parasites seen.

time. However, the three red deer remained carriers for at least 8 mo as determined by successful culture of the parasites from these animals at that time (data not shown).

The IFA test showed seroconversion of all three animals by day 14 (Table 5). The highest titers of antibody activity were against the homologous antigen, but cross-reactivity was also evident (Table 5). Immunoprecipitation analysis revealed the presence of shared antigens as well as antigens specific to the individual *Babesia* isolate (Fig. 2). Two antigen bands specific for the caribou *Babesia* sp. isolate migrating at 31.5 and 30.5 KDa, an antigen band migrating at 38.5 KDa specific for the elk *Babesia* sp. isolate, and an antigen band migrating at 36.5 KDa that was shared by the elk *Babesia* sp. isolate and *B. odocoilei* are indicated in Figure 2.

The blood sample from the seven-point male deer resident at the Minnesota Zoological Garden was positive for intraerythrocytic *B. odocoilei* after 3 days in culture. No *B. odocoilei* parasites were observed in cultures from the younger white-tailed deer during the cultivation period.

Amplification of the *Babesia* spp. SSU rRNA gene from *B. divergens*, *B. odocoilei*-E, *B. odocoilei*-B and the caribou and elk *Babesia* sp. isolates resulted in a single PCR product for each banding at approximately 1,800 base pairs (not shown). Sequence analysis showed the *B. divergens* and *B. odocoilei* SSU rRNA genes to be

TABLE 5. Indirect fluorescent antibody test of red deer sera collected 14 days post inoculation assayed against *Babesia odocoilei*-E, and the *Babesia* isolates from the elk and caribou. Results are expressed as reciprocal titers.

Antigen	Antiserum		
	R87∝Caribou *Babesia* isolate	R89∝Elk *Babesia* isolate	R92∝*Babesia odocoilei*-E
Caribou *Babesia* isolate	320	40	80
Elk *Babesia* isolate	40	160	80
Babesia odocoilei-E	40	No reaction	160

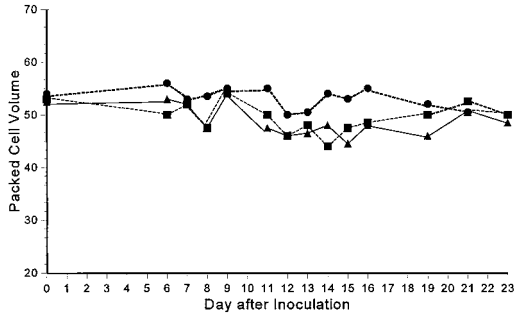

FIGURE 2. Individual red deer packed cell volumes after inoculation with either the caribou *Babesia* sp. isolate (–■–); the elk *Babesia* sp. isolate (–▲–); or *Babesia odocoilei*-E (–●–).

1724 and 1723 base pairs in length, respectively (Fig. 3). All of the cervine babesia isolates possessed SSU rRNA gene sequences identical to that of *B. odocoilei*-E (submitted to the GenBank database as *B. odocoilei*, Genbank Accession No. U16369), which differed at 32 positions from that of *B. divergens* (GenBank Accession No. U16370) (Fig. 3).

The *B. divergens* and *B. odocoilei* SSU rRNA gene sequences were aligned with appropriate SSU rRNA gene sequences of *Babesia* and *Theileria* spp. from the Ribosomal Database Project (RDP) (Larsen et al., 1993) and phylogenetic trees inferred by the NEIGHBOR distance method (Fig. 4), FITCH (not shown), DNAPARS (not shown), and fastDNAml (not shown) algorhythms. In each case, *B. divergens* and *B. odocoilei* form a closely related pair within the clade composed of "true" *Babesia* spp. (*Babesia* sensu stricto), i.e., those known to be transovarially tick transmitted and not having an intra-lymphocytic stage. The robustness of the inferred NEIGHBOR, FITCH, and DNAPARS trees were tested by 100 bootstrap resamplings, which consistently supported (100 out of 100 samples) the positions of *B. divergens* and *B. odocoilei*. The NEIGHBOR tree topology is presented as representative of the positioning of *B. divergens* and *B. odocoilei* within the *Babesia* sensu stricto clade (Fig. 4).

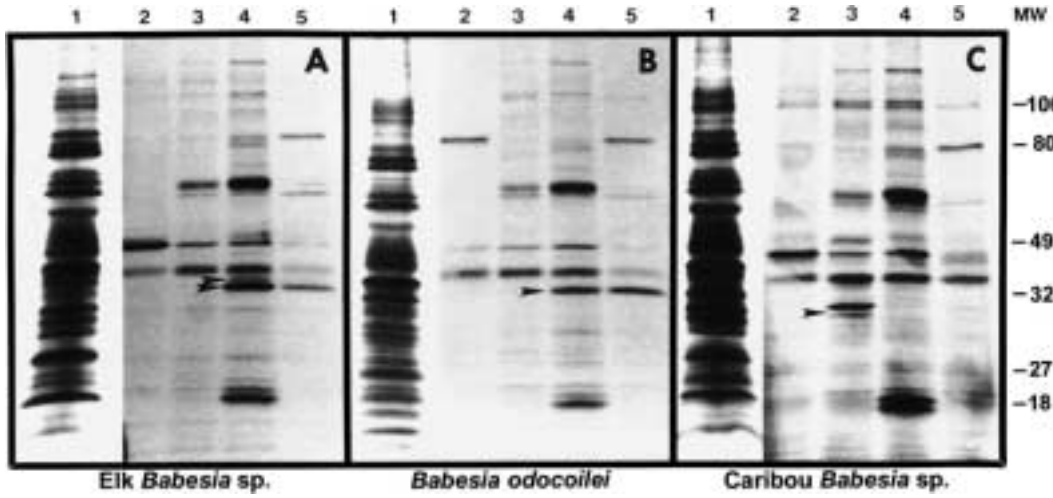

FIGURE 3. Immunoprecipitation analysis of the elk *Babesia* sp. (A), *Babesia odocoilei* (B), and the caribou *Babesia* sp. (C). In A, B, and C, lane 1 shows total radiolabelled protein for the respective isolate, lane 2 shows reactions with preimmune red deer R89 serum, lane 3 shows reactions with red deer R87 antiserum to the caribou *Babesia* sp., lane 4 shows reactions with red deer R89 antiserum to the elk *Babesia* sp., and lane 5 shows reactions with red deer R92 antiserum to *Babesia odocoilei*-E. A. The elk *Babesia* sp. immunoprecipitation shows a specific elk *Babesia* sp. antigen band at 38.5 KDa (arrow, lane 4). Arrows in lanes 4 and 5 show a 36.5 KDa antigen band recognized by both elk *Babesia* sp.- and *B. odocoilei*-E-specific antisera. B. *Babesia odocoilei* immunoprecipitation shows a 36.5 KDa antigen band recognized by both elk *Babesia* sp.- and *B. odocoilei*-E-specific antisera (arrows, lanes 4 and 5). C. The caribou *Babesia* sp. immunoprecipitation shows a caribou *Babesia* sp. specific antigen doublet bands at 30.5 and 31.5 KDa (arrow, lane 3).

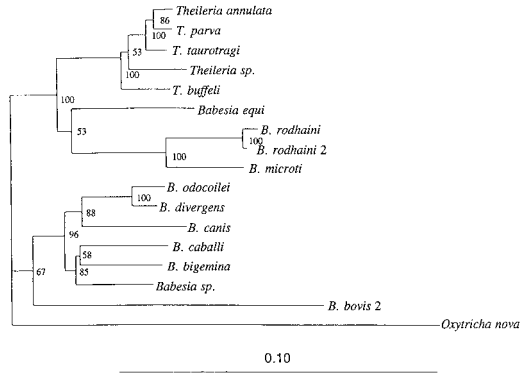

FIGURE 4. NEIGHBOR phylogenetic tree constructed from aligned SSU rRNA gene sequences of *Babesia* spp. and *Theileria* spp. with *Oxytricha nova* as the outgroup. The elk and caribou *Babesia* spp. SSU rRNA gene sequences and *Babesia odocoilei*-Engeling and *Babesia odocoilei*-Brushy Creek SSU rRNA gene sequences were all identical and are included in the tree as a single sequence for *Babesia odocoilei.* Relative evolutionary distances are represented by the horizontal distances between nodes of the tree. The scale bar corresponds to 10 changes per 100 nucleotide positions. Node values indicate the percentage of times that node was supported after 100 bootstrap resamplings.

DISCUSSION

This study suggests that in addition to white-tailed deer, caribou, and elk are hosts for *B. odocoilei.* The two *Babesia* sp. isolates from a caribou and an elk were indistinguishable from *B. odocoilei* of white-tailed deer on the basis of in vivo infections in red deer and SSU rRNA gene sequences. Although some antigenic variation was noted among the three *Babesia* spp., shared antigens also were evident by both IFA and immunoprecipitation methods.

The observed antigenic variation among these isolates and *B. odocoilei* indicated that traditional immunological methods of characterizing organisms were not able to unequivocally demonstrate whether the caribou *Babesia* sp. and the elk *Babesia* sp. represented the same species. Thus, DNA analysis was implemented to resolve the questions of relatedness and identity of these three parasite isolates. To this end, nucleotide sequence determination of the gene encoding the small-subunit ribosomal RNA was employed. Sequence analysis of the coding regions for the SSU rRNAs has gained widespread acceptance for molecular systematics, as shown with other *Babesia* spp. and the closely related hemoprotozoan parasites, *Theileria* spp., as well as other protozoa (Allsopp et al., 1993, 1994; Allsopp, 1994; Ellis et al., 1992; Gajadhar et al., 1991). In the current study, sequence analysis of the full length SSU rRNA gene showed that both the Brushy Creek and the Engeling *B. odocoilei* isolates from white-tailed deer had identical gene sequences. Further, the *Babesia* sp. isolates from the caribou and elk also possessed the same SSU rRNA gene nucleotide sequences as *B. odocoilei.* Thus identical SSU rRNA gene nucleotide sequences were confirmed in isolates from four geographic locations and from three different cervine hosts.

Babesia divergens was included in the SSU rRNA gene sequence analysis because phenotypic similarities between *B. divergens* and *B. odocoilei* have previously been noted (Gray et al., 1991) and both species parasitize deer (Gray et al., 1990). We now add a genetic aspect to the similarities found between these two parasite species. Alignment of the *B. divergens* SSU rRNA gene sequence with that of *B. odocoilei* shows that the respective SSU rRNA gene sequences differ at only 32 of more than 1,700 nucleotide positions. The phylogenetic trees inferred from hemoparasitic SSU rRNA gene sequences consistently paired *B. divergens* and *B. odocoilei* together within the group consisting of *Babesia* spp. sensu stricto (those *Babesia* spp. transovarially tick transmitted and that multiply only within the erythrocytes of their mammalian hosts). The observed pairing of *B. divergens* and *B. odocoilei* was well-supported by bootstrap analysis of the inferred tree. To further test the robustness of the inferred relationships, phylogenetic trees were also constructed using maximum likelihood, parsimony, and Fitch-Margoliash and least-squares dis-

tance algorhythms (data not shown). All methods placed *B. odocoilei* branched together with *B. divergens* in the "true" *Babesia* clade.

Babesia odocoilei is generally considered a nonpathogenic parasite of white-tailed deer (Perry et al., 1985). However, the elk and caribou *Babesia* sp. isolates were acquired as a direct result of acute babesiosis in their respective hosts. Since elk or caribou were not available, we attempted to fulfill Koch's postulates by using red deer to which the North American elk is a closely related subspecies. However, neither the elk nor caribou *Babesia* sp. isolates caused clinical disease in the red deer, although the animals did become carriers of the parasites. Each red deer demonstrated a similar mild response to infection regardless of the isolate inoculated, including the Engeling *B. odocoilei* isolate. Further, this mild response was consistent with previous observations when spleen-intact white-tailed deer were inoculated with *B. odocoilei*-infected blood or cultured parasites (Waldrup, 1991). A slight decrease in PCV in *B. odocoilei*-inoculated white-tailed deer between days 7 and 13 was the only clinical sign noted in those studies (Waldrup, 1991), which was consistent with the slight drop in PCV noted between days 12 and 14 after inoculation of the red deer. All three red deer became carriers of the respective parasites and remained carriers for at least eight months as confirmed by culture reisolation of the parasites. Previously, a white-tailed deer experimentally infected with *B. odocoilei* remained a carrier for ten months after inoculation as confirmed by culture reisolation of the parasite (Waldrup, 1991).

From the present study, we conclude that the *Babesia* sp. isolates from the caribou and elk are, in fact, *B. odocoilei*. The source of infection of the caribou at the Minnesota Zoological Garden has been speculative, as no other animals on the premises were affected (Holman et al., 1994b; Petrini et al., 1995). Our results suggest that the source of infection might be the white-tailed deer population resident at the Minnesota Zoological Gardens since we cultured *B. odocoilei* from a white-tailed deer in residence at the zoo at the time that the caribou became parasitemic (Holman et al., 1994b, Petrini et al., 1995). No ectoparasites were found on the caribou at the time it was clinically affected (Petrini et al., 1995). However, the incubation period following *B. odocoilei* transmission by *Ixodes scapularis* is at least 6–10 days (Waldrup et al., 1990; Waldrup, 1991), so at the time clinical signs were observed in the caribou, the vector ticks probably had detached from the animal. The potential for vector ticks from reservoir wild animals to access the zoo-housed animals exists. Ixodid ticks occur in the area and there are no physical barriers to prevent the entry of ticks onto the zoo premises.

The possibility of white-tailed deer harboring *B. odocoilei* infective for other as yet undiscovered mammalian hosts is of concern, particularly since the organism may be considered potentially pathogenic in compromised hosts. Intensive management and marketing practices may serve as stressors and thus expose animals to opportunities for disease manifestation and transmission. Also of concern is that the vector tick, *Ixodes scapularis*, is ubiquitous throughout much of eastern and central North America. The presence of unidentified reservoir animals and vector ticks, coupled with increased movement of nondomestic ungulates due to surging interest in ranching and farming of exotic species, raises the possibility of exposing naive susceptible animals to endemic environments or, alternatively, introducing *B. odocoilei* to native wildlife. Either situation could result in disease in a susceptible population. The potential impact of reservoir infection is of importance to the growing industry of deer and antelope ranching and to other handlers of exotic or native wildlife, including zoological parks, and underscores the risks involved in the introduction of outside animals to a herd.

A lack of reliable tests for accurate diagnoses of infectious diseases, including babesiosis, in exotic ungulates is a major problem facing the deer farming industry. The undetected presence of hemoparasites and their vectors may threaten not only the deer and exotic livestock industry, but domestic livestock as well. The information gained through this study can be applied to future research into suitable methods of diagnosis. Cultured parasites might be applied in IFA tests developed for serological determination of exposure to the parasites. Epidemiological surveys based on IFA tests using banks of cervine sera routinely obtained for mandated tuberculosis testing would provide information concerning which farmed exotic wildlife ruminants are susceptible to these *Babesia* isolates. Lastly, DNA-based assays derived from SSU rRNA gene sequences may be valuable in diagnosis and identification of carrier animals and for the specific identification of new isolates.

ACKNOWLEDGMENTS

The authors thank D. Melendy and A. Johnson for excellent technical assistance. This research was supported in part by the Texas Agricultural Experiment Station (Project H-6261).

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Received for publication 21 April 1999.