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NEW RUMINANT HOSTS AND WIDER GEOGRAPHIC RANGE IDENTIFIED FOR *BABESIA ODOCOILEI* (EMERSON AND WRIGHT 1970)

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ABSTRACT: *Babesia odocoilei* was found to infect two previously unknown host species, desert bighorn sheep (*Ovis canadensis nelsoni*) and musk oxen (*Ovibos moschatus*), both of which are members of the family Bovidae. Previously, *B. odocoilei* has been reported in only Cervidae hosts. New geographic regions where *B. odocoilei* infections have not been reported previously include Pennsylvania and New York, where fatal babesiosis has occurred in reindeer (*Rangifer tarandus tarandus*); New Hampshire, where elk (*Cervus elaphus canadensis*) have been affected; and California, home of the infected desert bighorn sheep. Infection with *B. odocoilei* in these hosts was confirmed by parasite small subunit ribosomal RNA gene sequence analysis. A serosurvey for *B. odocoilei* antibody activity in New Hampshire showed prevalence rates of 100% at two elk farms and 12% at another farm. Control of potential vector ticks, *Ixodes scapularis*, especially when translocating livestock, is imperative to prevent outbreaks of babesiosis in managed herds of potential host species.

Key words: *Babesia odocoilei*, babesiosis, Bovidae, Cervidae, desert bighorn sheep, musk ox, small subunit ribosomal RNA gene.

INTRODUCTION

Babesia odocoilei causes acute, often fatal babesiosis in elk (*Cervus elaphus canadensis*), reindeer (*Rangifer tarandus tarandus*), and caribou (*Rangifer tarandus caribou*) (Holman et al., 2000, 2003). Outbreaks in farmed herds and, in the case of the caribou, in a zoo have been reported in Indiana, Minnesota, Texas, and Wisconsin (Holman et al., 2000, 2003; Gallatin et al., 2003).

Babesia odocoilei is a tick-borne, intraerythrocytic, apicomplexan parasite that is known to infect cervids. First isolated as an unknown *Babesia* sp. from white-tailed deer (*Odocoileus virginianus*) in Texas, the parasite was later named *B. odocoilei* (Emerson and Wright, 1968, 1970). The organism was not transmissible to sheep, goats, or splenectomized calves, but it caused severe anemia and, sometimes, death in splenectomized deer (Emerson and Wright, 1968; Perry et al., 1985; Waldrup,

1991). Although *B. odocoilei* infections may contribute to decreased body condition in free-ranging deer, experimental inoculation of spleen-intact white-tailed deer and red deer (*Cervus elaphus elaphus*) resulted in benign infections (Emerson and Wright, 1968; Waldrup, 1991; Holman et al., 2000). *Babesia odocoilei* parasitemias are found in healthy immature deer, indicating that enzootic stability may exist (Perry et al., 1985). To date, *B. odocoilei* has not been reported in mammalian hosts outside of the family Cervidae.

Experimental transstadial transmission of *B. odocoilei* in white-tailed deer by *Ixodes scapularis* ticks has been demonstrated; however, transovarial transmission and competent vector ticks have not been shown (Waldrup et al., 1990). *Dermacentor albipictus* ticks were found on elk (*C. e. canadensis*) infected with *B. odocoilei* (Holman et al., 1994, 2000). This tick species, however, is not a confirmed vector for *B. odocoilei*. Moreover, this outbreak occurred

within the geographic distribution of *I. scapularis* on a farm near Del Rio, Texas, USA. *Babesia odocoilei* infections in various deer hosts have been reported in other regions within the *I. scapularis* geographic range, including eastern Texas and the Texas post oak savannah, Oklahoma, Minnesota, Wisconsin, Indiana, Virginia, and Massachusetts (Emerson, 1969; Waldrup et al., 1989a, b; Perry et al., 1985; Goethert and Telford, 2003). In addition, *B. odocoilei* has been identified molecularly in *I. scapularis* ticks in Maine, Massachusetts, and Wisconsin (Armstrong et al., 1998).

Gene sequence analysis of the small subunit ribosomal RNA (SSU rRNA) gene was used to definitively characterize parasites as *B. odocoilei* from infections in animals in New Hampshire, New York, Pennsylvania, and California. Furthermore, this report identifies two members of the family Bovidae, desert bighorn sheep (*Ovis canadensis nelsoni*) and musk oxen (*Ovibos moschatus*), as additional vertebrate hosts naturally infected by this parasite.

MATERIALS AND METHODS

Parasites

The desert bighorn sheep isolate (CA BH), which was obtained from free-ranging, clinically normal animals in the San Bernardino Mountains, California, has been described previously (Goff et al., 1993). A bull elk (NH Elk) in New Hampshire (Farm 1: 42°42'N, 71°10'W) became ill in October 2002 with clinical babesiosis. Three cases of fatal babesiosis in reindeer occurred during 2003 on farms in New York (42°42'N, 73°36'W; designated as NY R1 and NY R2, respectively) and Pennsylvania (39°42'N, 76°19'W; designated as PA R). Two musk oxen (MN MO1 and MN MO2) housed at the Minnesota Zoological Garden, Apple Valley, Minnesota, USA, developed acute cases of fatal babesiosis in September 2003 and November 2003, respectively. Subsequently, two additional musk oxen at the zoo (MN MO3 and MN MO4) tested positive for *B. odocoilei* by specific polymerase chain reaction (PCR) testing but did not show clinical signs.

Parasite DNA was obtained from an original cryopreserved blood stabilate of the CA BH isolate (Goff et al., 1993), from blood samples from the reindeer (kindly provided by C.

Cripps, Battenkill Veterinary Bovine PC, Greenwich, New York, USA, and J. A. Cohagan, Muddy Creek Veterinary Service, Fawn Ridge, Pennsylvania, USA) and musk oxen, and from cultured parasites from the NH Elk (Table 1). The parasite culture was established as described previously, and DNA was extracted from the cultures when the parasitemia was 10% (Holman et al., 2003).

Parasite morphology

Giemsa-stained blood smears (NY reindeer, PA reindeer, MN musk ox, and NH elk) or Giemsa-stained erythrocyte smears from culture (CA bighorn sheep parasite) were examined under oil at 1,000× magnification. Parasites at the periphery of the erythrocyte (accolé position), small paired piriforms, and erythrocytes containing four or more parasites are characteristic of *B. odocoilei* (Holman et al., 1988; Gray et al., 1991).

Indirect fluorescent antibody test

Elk serum samples collected from 15 cohort elk on Farm 1, 17 elk on Farm 2 (43°8'N, 70°57'W), and 26 elk on Farm 3 (43°37'N, 72°19'W) in New Hampshire were tested for *B. odocoilei* antibody activity by the indirect fluorescent antibody test using standard protocols (Goff et al., 1993). Fluorescein-labeled protein G (Sigma) served as the conjugate (Goff et al., 1993). Antigen slides were prepared previously from cultures of *B. odocoilei* in white-tailed deer erythrocytes and stored frozen at -20 C until use. All serum samples and appropriate positive- and negative-control samples were tested at a 1:80 dilution in phosphate-buffered saline (pH 7.2). All tests were observed under oil immersion at 1,000× magnification on a Labophot-2 model microscope (Nikon, Tokyo, Japan) and scored as positive or negative reactions.

SSU rRNA gene sequence analysis

Genomic DNA was purified from each sample using a standard phenol-chloroform extraction method facilitated by the use of Phase Lock Gel tubes (Phase Lock Gel System, Eppendorf AG, Hamburg, Germany). The SSU rRNA genes were amplified from approximately 50–100 ng of template genomic DNA using 1 pmol each of primers A and B (Fig. 1 and Table 1) (Sogin, 1990) in a 25-236l reaction volume (Advantage 2 PCR Enzyme System, BD Biosciences, Palo Alto, California) as described previously (Holman et al., 2003). PCR products were separated by electrophoresis through a 1% (w/v) agarose gel alongside a 100 BP mark-

TABLE 1. Small subunit ribosomal RNA (SSU rRNA) gene acquisition details for each *Babesia* sp. isolate including source of DNA, primers used to obtain the SSU rRNA gene amplicon, amplicon size, whether the DNA sequence was obtained directly from the amplicon or from plasmid clones, number of base pairs sequenced, and primers used for sequencing.

Isolate ^a	DNA source	Amplicon primers	Amplicon size	DNA sequenced	No. of bp sequenced	Sequencing primers	GenBank accession no.
CA bighorn sheep	Blood stabilate	A/B	1,723	Amplicon	1,605	528F, AN, BN	AY661502
NH elk	Culture	A/B	1,723	Amplicon	1,608	528F, AN, BN	AY661503
NY reindeer 1	Blood	A/B	1,723	Clone	1,723	528F, M13F, M13R	AY661504
NY reindeer 2	Blood	A/B	1,723	Clone	1,723	528F, M13F, M13R	AY661505
PA reindeer	Blood	A/B	1,723	Clone	1,723	M13F, M13R	AY661506
MN musk ox 1	Blood	A/B	1,723	Amplicon	1,605	AN, BN	AY661507
MN musk ox 2	Blood	A/B	1,723	Clone	1,723	M13F, M13R	AY661508
MN musk ox 3	Blood	AN/BN	1,658	Clone	1,658	528F, M13F, M13R	AY661509
MN musk ox 4	Blood	AN/BN	1,658	Clone	1,658	528F, M13F, M13R	AY661510

^a CA = California; MN = Minnesota; NY = New York; PA = Pennsylvania.

er (Invitrogen Corp., Carlsbad, California, USA) stained with ethidium bromide and the bands visualized by ultraviolet transillumination. In the case of the MN MO3 and MN MO4 isolates, a nested PCR was performed as described above but using 1 μ l of the primary PCR product as template. The nested primer set AN and BN was designed just internal to primers A and B, with primer AN located approximately 30 bases downstream of the 5' end and BN located approximately 25 bases upstream of the 3' end (Fig. 1 and Table 1). The DNA isolation, PCR master mix preparation, template addition to the PCR master mix aliquots, and cloning procedures were conducted in separate locations in hoods with dedicated equipment and materials for each step to prevent cross-contamination of samples. Distilled water template controls were included in each PCR experiment to ensure that no carryover contamination occurred. For nested PCR, two water controls were included, one using the primary water control as template and a second with new water as template. Plasmid DNA containing the *B. odocoilei* SSU rRNA gene insert served as a positive control. Each isolate was handled at a different time frame to prevent cross-contamination.

The CA BH, NH elk, and MN MO1 SSU rRNA genes were directly sequenced from the primary SSU rRNA gene products. The SSU rRNA gene amplicons from the remaining isolates were cloned before sequencing as described previously (Holman et al., 2003). The full SSU rRNA gene nucleotide sequences were obtained from at least three plasmid clones by sequencing with primers 528F, M13 Forward (-20), and M13 Reverse. Primers 528F, AN, and BN were used to directly sequence purified PCR amplicons. Primers used for sequencing each product are listed in Table 1. Gene-specific primer sequences are shown in Figure 1. All sequencing reactions and automated sequencing were performed by services either at the Gene Technologies Laboratory (Department of Biology) or the DNA Technologies Laboratory (Department of Veterinary Pathobiology) of Texas A&M University, College Station, Texas, USA.

The obtained sequences were analyzed using Sequencher 3.11 software (Gene Codes Corporation, Inc., Ann Arbor, Michigan, USA). A consensus sequence for each cloned isolate was determined. BLAST similarity searches (Altschul et al., 1990) were performed for all SSU rRNA gene sequences obtained (GenBank database, National Center for Biotechnology Information, National Institutes of Health, Bethesda, Maryland, USA). An alignment of the obtained direct and consensus sequences was

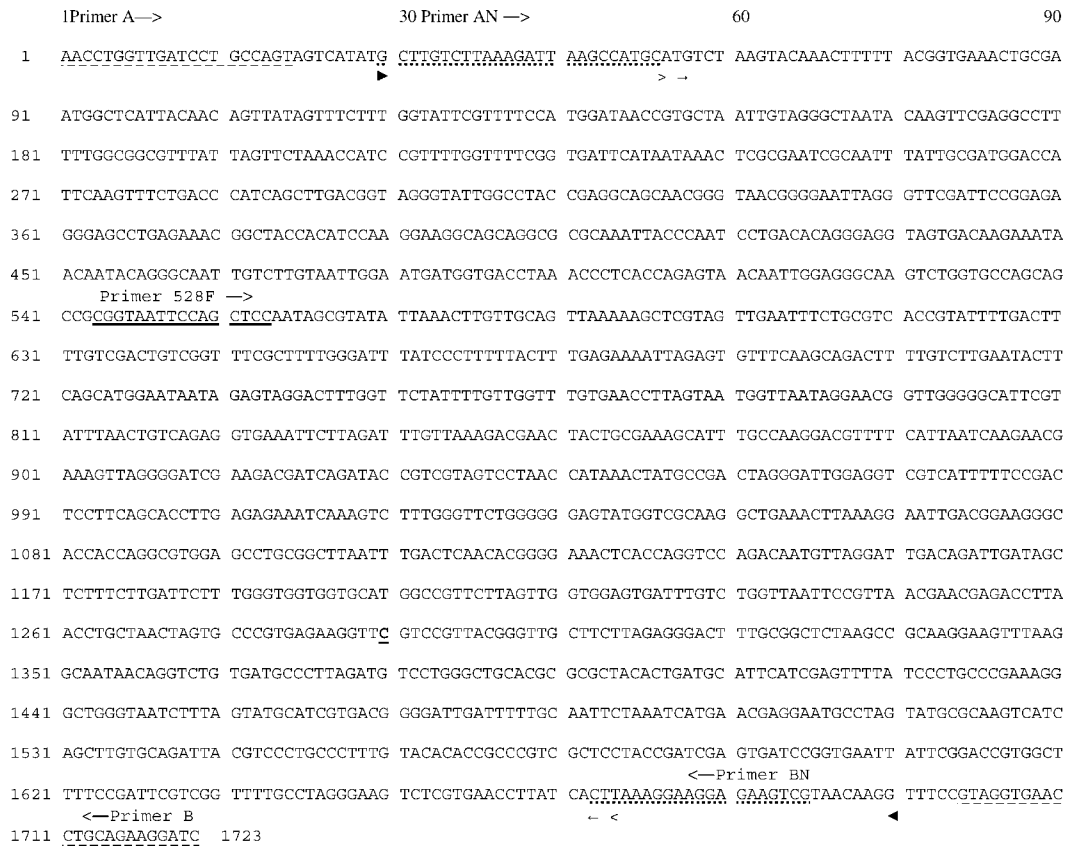


FIGURE 1. *Babesia odocoilei* small subunit ribosomal RNA (SSU rRNA) gene sequence with denotation of oligonucleotide primer sequences and positions. The SSU rRNA gene sequence from the Pennsylvania (PA) and New York (NY) reindeer and from Minnesota (MN) musk ox 2 isolates is identical to the gene sequence of *B. odocoilei* (GenBank accession no. U16369). The SSU rRNA gene fragment sequence for the MN musk oxen 3 and 4 isolates begins at position 30 and ends at position 1,687 (▶, ◀). The New Hampshire (NH) elk isolate sequence begins at position 55 and ends at position 1,662 (>, <). The California (CA) bighorn sheep and MN musk ox 1 isolate sequences begin at position 56 and end at position 1,660 (→, ←). All sequences were identical to that of U16369 except for a single base substitution in the CA bighorn sheep isolate sequence (thymidine for a cytosine at position 1,290; bold type, underlined). The positions of primers A and B are indicated by broken underlining, of primers AN and BN by dotted underlining, and of 528F by bold underlining. Primer orientation is indicated by arrows.

created using the ClustalW 1.8 Program (<http://searchlauncher.bcm.tmc.edu/multi-align/multi-align.html>).

RESULTS

All the babesia organisms from the bighorn sheep, reindeer, elk, and musk oxen are morphologically similar to each other (Fig. 2). All are small piroplasmids (<2.5 μm in length for paired piroplasmids) (Levine, 1985). They frequently are seen as tetrads,

and they often are located peripherally in the host erythrocyte, as described previously for *B. odocoilei* (Holman et al., 1988, Gray et al., 1991). Voucher specimens from each host species were deposited at the National Parasite Collection, Beltsville, Maryland, USA, as follows: MN MO1, USNCP 95152 *B. odocoilei* ex *O. moschatus*; NH Elk, USNCP 95153 *B. odocoilei* ex *C. e. canadensis*; NY R1, USNCP 95154 *B. odocoilei* ex *R. t. tarandus*; PA R,

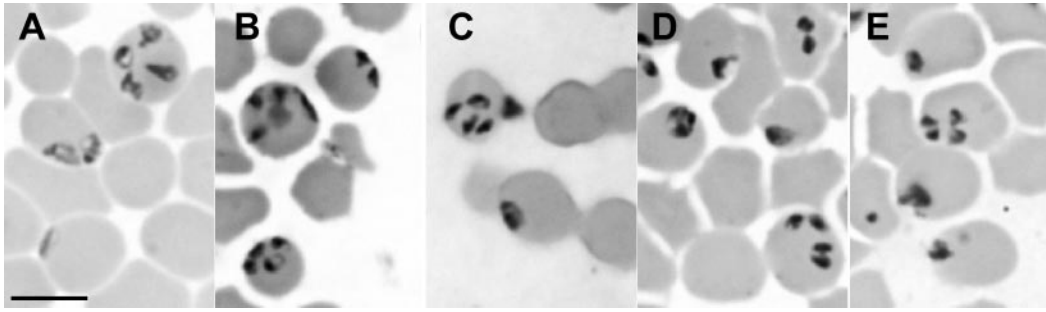


FIGURE 2. *Babesia odocoilei* intraerythrocytic forms from various hosts. A. California (CA) desert bighorn sheep parasites cultured in bighorn sheep erythrocytes. B. Infected blood ex New York (NY) reindeer. C. Infected blood ex Minnesota (MN) musk ox. D. Infected blood ex New Hampshire (NH) elk. E. Infected blood ex Pennsylvania (PA) reindeer. Giemsa stain. Bar = 5 μ m.

USNCP 95155 *B. odocoilei* ex *R. t. tarandus*; and CA BH, USNCP 95156 *B. odocoilei* ex *O. c. nelsoni*.

The SSU rRNA gene fragments of 1,723 or 1,658 bp in length were obtained using primers A and B or nested primers AN and BN, respectively (Table 1). Sequence details for each isolate are given in Table 1. A consensus sequence from a minimum of three cloned sequences per isolate was deposited into the GenBank database (accession nos. as follows: NY R1, AY661504; NY R2, AY661505; PA R, AY661506; MN MO2, AY661508; MN MO3, AY661509; MN MO 4, AY661510) (Table 1). The sequences obtained directly for CA BH, NH Elk, and MN MO1 were deposited in the GenBank database under accession numbers AY661502, AY661503, and AY661507, respectively (Table 1).

The resulting SSU rRNA sequences for all nine isolates were aligned with the 1,723-bp SSU rRNA sequence for *B. odocoilei* in the GenBank database (Engeling Texas white-tailed deer isolate, GenBank accession no. U16369) (Holman et al., 2000) (graphic alignment not shown for present study). The consensus SSU rRNA gene sequences from the three reindeer, the New Hampshire elk, and the four musk oxen isolates all showed 100% identity to the *B. odocoilei* U16369 sequence (Fig. 1). The CA BH isolate showed 99.9% identity to U16369, with a thymidine sub-

stituted for a cytosine at position 1290, as indicated in Figure 1.

A serosurvey of elk on three farms in New Hampshire revealed prevalence rates of 100% on Farm 1 (15/15 positive), 100% on Farm 2 (17/17 positive), and 12% on Farm 3 (3/26 positive) for indirect fluorescent antibody activity to *B. odocoilei*.

DISCUSSION

Originally described and named as a parasite of white-tailed deer, *B. odocoilei* has since been described in several members of the family Cervidae, including elk (*C. e. elaphus*), caribou (*R. t. caribou*), and reindeer (*R. t. tarandus*) (Emerson and Wright, 1968, 1970; Holman et al., 2000, 2003; Gallatin et al., 2003). For the most part, the expansion of known hosts has resulted directly from outbreaks of acute babesiosis in farmed deer (Holman et al., 1994, 2003; Petrini et al., 1995; Gallatin et al., 2003). In the case of elk, screening of cohort animals in a herd subsequent to an outbreak of acute fatal babesiosis showed that subclinical infection may exist, providing a reservoir of infection (Gallatin et al., 2003). In the case of the caribou, however, subclinical infections were not found among cohort caribou; rather, white-tailed deer resident in near proximity appeared to be the reservoir of infection (Petrini et al., 1995; Holman et al., 2000).

The absence of carriers among the car-

ibou raises the question of whether caribou are more prone to severe clinical disease after infection. Because no further cases in caribou have been reported, this question cannot be answered unequivocally. All cases in reindeer, a sister subspecies to caribou (*R. t. tarandus* and *R. t. caribou*, respectively), reported to date (including the three current cases and a previous case described by Holman et al., 2003) presented as acute babesiosis, however, and were rapidly fatal. A previous case involved a 7-mo-old reindeer, which strongly suggests that the phenomenon of age-related immunity does not play a role in *B. odocoilei* infections in reindeer. With some *Babesia* spp., most notably the agents of bovine and equine babesiosis, young animals are susceptible to infection without clinical manifestations but mount an immune response directed against the infecting parasite (Levine, 1985). Thus, a carrier state is established without clinical disease. As long as the animal maintains a carrier infection, it is refractory to disease unless stressed or otherwise immune compromised. This state may exist in elk, because subclinical carriers have been identified (Gallatin et al., 2003).

With the discovery of *B. odocoilei*-infected reindeer in New York and Pennsylvania and the infected elk in New Hampshire, the known range of the parasite in mammalian hosts is extended to New England states beyond Massachusetts (Goethert and Telford, 2003). Serosurveys of elk herds at different localities in New Hampshire showed a prevalence rate of 100% at two and a low prevalence of exposure (12%) at the third. These data suggest that *B. odocoilei* is endemic in New Hampshire. Moreover, molecular epidemiologic studies demonstrated *B. odocoilei* DNA in *I. scapularis* ticks as far north as Maine (Armstrong et al., 1998). The known range of *B. odocoilei* will, in time, likely correlate with the leading territorial edge of the vector tick distribution.

Babesia spp. parasites of clinically normal, free-ranging desert bighorn sheep (*O.*

c. nelsoni) in the San Bernardino Mountains near Los Angeles, California, USA, were reported in 1993 (Goff et al., 1993; Thomford et al., 1993). The *B. odocoilei*-like parasites described in a Rocky Mountain bighorn lamb (*Ovis canadensis canadensis*) subinoculated with pooled blood from the free-ranging animals (Goff et al., 1993) were confirmed in the present study by molecular analysis to be *B. odocoilei*. Three additional morphologically distinct small piroplasm isolates, designated as BH1, BH2, and BH3, were obtained by cultivation of infected blood from desert bighorn sheep (Thomford et al., 1993). Both BH1 and BH2 were similar in size to *B. odocoilei*, but they were not found in the accolé position characteristic of *B. odocoilei*. Unlike *B. odocoilei*, BH3 is a large piroplasm (piriform average length, 2.6 μ m) (Thomford et al., 1993). Subsequent SSU rRNA gene sequencing of BH1 and BH3 showed them to be divergent from *B. odocoilei* (Kjemtrup et al., 2000); the SSU rRNA gene sequence for BH2 has not been reported.

The definitive identification of the desert bighorn sheep *Babesia* sp. isolated by Goff et al. (1993) as *B. odocoilei* is significant, because this is the first recognition of a vertebrate host for this parasite outside the family Cervidae. Furthermore, this delineation expands the known geographic range of the hemoparasite to California, which is outside the geographic distribution of the known vector tick, *I. scapularis*.

The geographic range of *I. scapularis* includes the state of Florida in the southeastern United States north to the Canadian provinces of Nova Scotia and Prince Edward Island, west to North Dakota and South Dakota, and south to the Mexican state of Coahuila (Keirans et al., 1996), excluding the western coastal United States. In fact, using a predictive model, Brownstein et al. (2003) conclude that the California habitat is unsuitable for supporting *I. scapularis*. Therefore, an alternate vector tick species for *B. odocoilei* must be

active in California. *Ixodes* sp. ticks infested bighorn sheep in the geographic region from which the CA BH isolate was obtained (Goff et al., 1993). This observation suggests that *Ixodes pacificus* may vector *B. odocoilei* in California. Clinically affected elk infected with *B. odocoilei* in Texas were infested with *D. albipictus* ticks, a species that is widely distributed in California but concentrated in the central coastal and Sierra foothill areas of California (Holman et al., 1994; Furman and Loomis, 1984). The roles of these two tick species in the biology of *B. odocoilei* remain to be confirmed.

The addition of musk ox (*O. moschatus*) as another member of the family Bovidae that is susceptible to *B. odocoilei* infection is noteworthy. *Babesia odocoilei* is not transmissible to domestic sheep, goats, or splenectomized calves (Emerson and Wright, 1968). The fact that two members of the Bovidae family have now emerged as competent, naturally infected hosts suggests that other as-yet-unidentified host species may exist and indicates the potential for babesiosis outbreaks in previously unknown hosts.

Molecular tools may aid in predicting the future geographic distribution of *B. odocoilei* infections. Armstrong et al. (1998) showed molecular evidence of *B. odocoilei* in salivary glands of *I. scapularis* ticks in Wisconsin, Massachusetts, and Maine. In Wisconsin, *B. odocoilei* infections in elk and reindeer are documented, and *B. odocoilei* has been confirmed molecularly in white-tailed deer in Massachusetts (Goethert and Telford, 2003; Holman et al., 2003). To date there have been no reports of infected animals in Maine. The parasite is likely to emerge in additional animal hosts and geographic areas as it is introduced via translocation of infected animals into new environments or as naïve animals are introduced into endemic environments. Effective tick-control measures and judicious animal management practices should be implemented to pre-

vent transmission of this tick-borne pathogen to susceptible animals.

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