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Genotypically unique *Babesia* spp. isolated from reindeer (*Rangifer tarandus tarandus*) in the United States

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Abstract Two morphologically dissimilar *Babesia* spp. were cultured from reindeer (*Rangifer tarandus tarandus*) in Placer County, Calif. The smaller isolate, designated RD61, was morphologically similar to *Babesia odocoilei*. Serum from RD61-infected reindeer reacted equally strongly to *B. odocoilei* and RD61 parasites in the indirect fluorescent antibody (IFA) test. Small subunit ribosomal RNA (SSU rRNA) gene-sequence analysis showed 99.0% identity to that of *B. odocoilei*. The larger piroplasm, designated RD63, resembled larger babesia organisms, such as *Babesia caballi* and *Babesia bigemina*. Serum from RD63-infected reindeer also reacted with both *B. odocoilei* and RD61 parasites in the indirect fluorescent antibody test. The SSU rRNA gene showed 94.2% identity to that of *B. bigemina*. Further studies are needed to determine whether these parasites are the same as the *Babesia* spp. previously documented in Siberian reindeer.

Nucleotide sequence data reported in this paper have been submitted to the GenBank database under accession numbers AF411337 and AF411338

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Introduction

The first documentations of babesia in reindeer (*Rangifer tarandus tarandus*) were *Piroplasma tarandi rangiferis* in Russia in 1909 by Dr. S. Kertzelli (Chambers 1921) and *Francaiella tarandi rangifer* in 1929 by Yakimoff and Kolmakoff (cited by Nilsson et al. 1965). Although these two species were synonymized with *Theileria tarandi* in 1971 (Levine 1971), in 1988 Friedhoff noted that concurrent infections with theileria and babesia in reindeer were documented (Chambers 1921), and asserted that probably one *Theileria* species and at least one *Babesia* species occur in this host. The smaller babesia of reindeer is morphologically similar to *Babesia divergens*, a small piroplasm of cattle that has been shown experimentally to infect reindeer (Nilsson et al. 1965). A larger piroplasm, *Babesia jakimovi*, also has been described in field-infected reindeer (Nikol'skii et al. 1977).

In the United States, *Babesia odocoilei* was implicated in a case of fatal babesiosis in a zoo-housed caribou (*Rangifer tarandus caribou*) (Petrini et al. 1995; Holman et al. 2000). Originally reported as a parasite of white-tailed deer (*Odocoileus virginianus*), *B. odocoilei* is now known to infect both elk (*Cervus elaphus elaphus*) and caribou (Spindler et al. 1958; Emerson and Wright 1970; Holman et al. 1994). Like the European small piroplasm described in reindeer, *B. odocoilei* is morphologically similar to *B. divergens*. All three parasites share the morphological characteristic of frequently being located in the accolé position within the host erythrocyte.

The possible involvement of a *Babesia* sp. infection in the death of a young reindeer in California led to the current study. Post-mortem findings included large and small intraerythrocytic bodies suggestive of the presence of either *Anaplasma* sp. or *Babesia* sp. organisms. We describe herein two *Babesia* spp. isolates cultured from

cohort herd members and characterized by morphologic, serologic, and genetic analysis.

Materials and methods

Animals and blood samples

Four reindeer included in this study were members of a herd housed in Applegate (39:00:03 N and 120:59:29 W; altitude 2,000 feet), Placer County, Calif., located in the Western Sierra foothills. Three of the reindeer originated from Redmond, Wash., and had been resident in the herd since December 1996. These included a 5-year-old castrated male (reindeer 61) and two 2-year-old females (reindeer 62 and reindeer 63). The fourth animal, reindeer 2570, was a female approximately 5 months of age that originated from Hobbs, N.M.

The four reindeer were physically restrained and blood samples were drawn by jugular venipuncture on 20 January 1999. Blood collected in ethlenediaminetetraacetic acid (EDTA), serum samples, and unstained blood films from the reindeer were shipped cold overnight to Texas A&M University, College Station, Tex., USA. Upon arrival, the serum samples were aliquoted and stored at –70 °C until use, EDTA blood samples were immediately processed for in vitro culture as described below, and the blood films were fixed with methanol and Giemsa-stained for microscopic examination.

Babesia spp. in vitro cultivation

Babesia spp. cultures were initiated using a previously described method with minor modifications (Holman et al. 1998). The reindeer blood samples collected in EDTA were centrifuged at 500 *g* for 10 min at 10 °C to pellet the erythrocytes (RBC). After removal of the plasma and buffy coat, 0.3 ml RBC from the bottom of the tube was drawn into a pipet containing 1.0 ml Dulbecco's phosphate buffered saline (pH 7.4) containing 15 mM EDTA (DPBS/EDTA) and dispensed into a 2.0 ml microcentrifuge tube and mixed by gentle pipetting. The RBC were washed twice in DPBS/EDTA, by centrifugation at 600 *g* for 3 min at room temperature. After a final wash in DPBS without EDTA, the supernatant was removed and discarded. The RBC pellet was resuspended to 1.6 ml in medium B consisting of HL-1 medium (BioWhittaker) with 20% fetal bovine serum (Hyclone Laboratories, Inc, Logan, Utah), 2 mM L-glutamine, and 50 μg/ml gentamicin. Four wells of a 24-well culture plate received 0.4 ml of the cell suspension. Two wells received an additional 0.85 ml medium B; and two wells received an additional 0.85 ml medium A consisting of HL-1 medium supplemented with 20% fetal bovine serum, 1% HB101 supplement (volume/volume after reconstitution of lyophilized supplement according to manufacturer's instructions; Irvine Scientific, Santa Ana, Calif.), 2 mM L-glutamine, 200 μM sodium hypoxanthine (Zweygarth et al. 1995) and 16 μM thymidine (HT Supplement; GIBCO, Grand Island, N.Y.). The cultures were incubated at 37 °C in a humidified atmosphere of 2% oxygen, 5% carbon dioxide, and 93% nitrogen.

Cultures were fed daily by removal of 1 ml of the medium overlying the cell layer, followed by addition of 1 ml of appropriate fresh medium. Thin erythrocyte films were made daily from the cultures starting on day 4 after initiation. The films were methanol-fixed, Giemsa-stained, and evaluated to monitor the growth of parasites.

Individual intracellular parasites were measured from Giemsa-stained erythocyte films from culture, using a micrometer and viewed at 1,000× oil immersion. Average parasite size was calculated from ten measurements from ring forms, single parasites, and paired forms. For infected cells containing three or more parasites, eight measurements were taken.

Indirect fluorescent antibody test

The *B. odocoilei* antigen used in the indirect fluorescent antibody (IFA) test was previously prepared from primary cultures of *B. odocoilei*-infected elk erythrocytes (cultured in medium B as described above) at a parasitemia of 16.8% and stored at –20 °C until use. Antigen slides for RD61 parasites were prepared similarly as follows. Briefly, erythrocyte cultures were resuspended and centrifuged at 500 *g* for 10 min. The supernatant was removed and the cells resuspended in an equal volume of DPBS containing 0.1% ovalbumin. Antigen slides were prepared and allowed to dry overnight. Masking tape was affixed on the cell-side of the slides after they were thoroughly dry, and the slides were stored at –20 °C until use. At use, the slides were allowed to equilibrate at room temperature before the tape was removed.

Standard protocols were followed for the IFA procedure using fluorescein-labeled Protein-G (Sigma, St Louis, Mo., USA) as the conjugate (Goff et al. 1993). Sera from reindeer 61, 62, 63 and 2750 were tested at a dilution of 1:80 in phosphate buffered saline, pH 7.2. Appropriate positive and negative control sera were included. All tests were observed under 1,000× magnification on an American Optical model 120 epifluorescent microscope (American Optical, Scientific Instrument Division, Buffalo, N.Y., USA).

SSU rRNA gene-sequence analysis

Genomic DNA was purified from cultured parasites using standard phenol/chloroform extraction methods (Sambrook et al. 1989). *Babesia* spp. SSU rRNA genes were amplified from approximately 50 ng genomic DNA in a 20 μl volume containing 1 pmol each primer A and B (Sogin 1990), 5 mM KCl, 1 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$ and 0.4 U Taq polymerase. Negative controls contained no template DNA. The amplification parameters were as previously described for piroplasm SSU rRNA gene amplification (Allsopp et al. 1989), except that the extension step was progressively increased by 10 s/cycle in a PTC-200 Peltier Thermal cycler (MJ Research, Watertown, Mass., USA). The amplicons were separated by electrophoresis through a 1% agarose gel and visualized by staining with ethidium bromide.

The appropriately sized amplicons (approximately 1,800 bp) were directly ligated into the plasmid vector pCRII and INV F' One Shot competent cells transformed according to manufacturer's instructions (TA Cloning Kit; InVitrogen, San Diego, Calif., USA). Transformed clones were color-selected and plasmid DNA for each selected clone was purified from overnight broth cultures by a modified alkaline lysis procedure (QIAprep Spin Miniprep Kit; Qiagen, Valencia, Calif., USA). The plasmid DNA samples were then screened by sequencing the V4 variable region of the gene using primer 528F as previously described (Chae et al. 1998). Sequencing reactions were made using Dye Terminator Cycle Sequencing Ready Reaction (PE Applied Biosystem, Norwalk, Conn., USA), and sequencing was carried out in either an ABI PRISM model 373A or ABA model 377 automated sequencer with version 1.2.2 or version 2.1.1 software, respectively (Gene Technologies Laboratory, Institute of Developmental and Molecular Biology, Department of Biology, Texas A&M University, College Station, Tex., USA). Further SSU rRNA gene sequencing was similarly carried out using primers complementary to the T7 and m13 reverse promoter regions (Stratagene, La Jolla, Calif., USA) of the plasmid vector, and a series of previously described internal primers for SSU rRNA gene sequencing (Elwood et al. 1985). The sequences obtained in this study were subjected to BLAST searches (Altschul et al. 1990) by the GenBank database (National Center for Biotechnology Information, National Institute of Health).

Results

Reindeer

Three days after blood collection, reindeer 61 became ataxic and died 24 h later on 24 January 1999. On 27 January 1999, reindeer 2570 also died. Necropsy results

did not produce a definitive cause of death for either animal. There was no indication that *Babesia* spp. infection contributed to the deaths (California Veterinary Diagnostic Laboratory System, University of California, Davis, Calif.). No piroplasms were found on the Giemsa-stained blood films from the four reindeer. In some cases, dark-staining bodies were observed that might have been condensed parasites, but no clearly defined parasites were seen.

Babesia spp. cultures

Babesia sp. parasites were observed in cultured erythrocytes from reindeer 61 and 63 on the 4th day after initiation (Table 1). Reindeer 61 erythrocytes were parasitized with a rapidly proliferating *B. odocoilei*-like organism, which was designated RD61. RD61 occurred in various forms within individual erythrocytes, including rings, single piriforms, paired piriforms, or multiple parasites (Fig. 1A). The parasites ranged in size from 1.93 μm in diameter for rings to 1.20 μm in diameter by 2.04 μm in length for paired forms (Table 2). The multiple forms generally were a little smaller, averaging 1.12 μm in diameter by 2.0 μm in length.

A larger piroplasm was observed in reindeer 62 and 63 cultured erythrocytes (Fig. 1B, Table 1) and was designated RD63. This parasite was morphologically similar to the "large" babesias such as *Babesia bigemina*, *Babesia caballi* and *Babesia canis*. RD63 was observed as ring forms or as single or paired piriforms (Fig. 1B) and ranged in size from 2.58 μm in diameter for rings to 1.97 μm in diameter by 3.41 μm in length for paired forms (Table 2). Single parasites averaged 2.38 μm in diameter by 4.12 μm in length (Table 2). The parasites in reindeer 63 erythrocyte cultures remained at a low percent parasitemia throughout the culture period (Table 1). Only a few parasitized erythrocytes were seen in the cultures from reindeer 62 (Table 1).

RD61, the smaller parasite, continued to proliferate in culture until a parasitemia of 11.6% was achieved on day 7. At this time, the cultures were divided into aliquots for DNA extraction, parasite cryopreservation, and preparation of IFA antigen. RD63 parasite cultures were also terminated at 7 days and divided into aliquots for DNA extraction and parasite cryopreservation. IFA antigen slides were not prepared from this isolate because of the low RD63 parasitemia (3.25%).

Table 1 Culture results for small and large *Babesia* isolates from reindeer samples based on Giemsa-stained erythrocyte smears. *NPS* No parasites seen

Day	Medium	Reindeer			
		61	62	63	2570
4	A	+ small	NPS	+ large	NPS
	B	NPS	NPS	NPS	NPS
5	A	+ small	+ large	+ large	NPS
	B	+ small	NPS	+ large	NPS
6	A	+ small	NPS	+ large	NPS
	B	+ small	+ large	+ large	NPS
7	A	+ small	NPS	+ large	NPS
	B	+ small	NPS	+ large	NPS

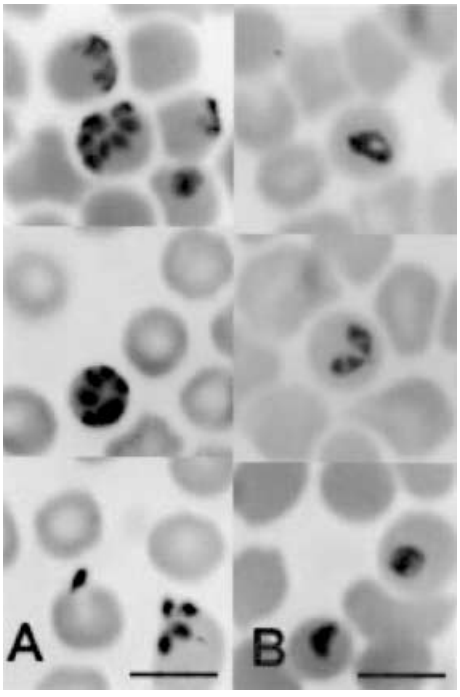


Fig. 1 Various piroplasm forms of RD61 (**A**) and RD63 (*B*) *Babesia* isolates from California reindeer. *Bars* 8 μm

IFA tests

Sera from reindeer 61, 62, 63, and 2750 were tested for antibody activity against the *B. odocoilei*-like reindeer RD61 parasite and against *B. odocoilei*. Serum from reindeer 61, 62, and 63 all reacted positively against the RD61 isolate, although there was a distinctly weaker reaction with reindeer 62 serum compared with the other two sera (Table 3). Reindeer 63 serum reacted more strongly against the RD61 isolate antigen than against the *B. odocoilei* antigen (Table 3). The serum from reindeer 2750 did not show any antibody activity against either antigen tested. *B. odocoilei* positive control serum reacted equally strongly against both antigens. The negative control serum did not have detectable antibody activity against either antigen (not shown).

SSU rRNA sequence analysis

Amplicons of approximately 1800 bp were obtained from both RD61 and RD63 isolates using primers A and B. BLAST searches by the GenBank database showed the nucleotide sequence of the cloned RD61 SSU rRNA gene amplicon to be most homologous to the *B. odocoilei* SSU rRNA gene sequence and to the SSU rRNA gene sequence of an unnamed *Babesia* isolate from a

Table 2 Measurements (μm) of different forms of RD61 and RD63 *Babesia* spp. organisms

Isolate	Parasite forms			
	Rings Diameter	Singles Diameter × length	Pairs Diameter × length	Multiples Diameter × length
RD61	1.93 ± 0.95	1.00 ± 0.35 × 2.38 ± 0.82	1.20 ± 0.25 × 2.04 ± 0.38	1.12 ± 0.24 × 2.00 ± 42
RD63	2.58 ± 0.57	2.38 ± 0.29 × 4.12 ± 0.77	1.97 ± 0.22 × 3.41 ± 0.59	Not done

Table 3 Indirect fluorescent antibody test of reindeer sera reacted with *B. odocoilei* and the RD61 *Babesia* isolate. Results are expressed as the presence or absence of relative fluorescence

Antigen	Antiserum				
	∝-*B.odocoilei*	Reindeer 61	Reindeer 62	Reindeer 63	Reindeer 2570
B. odocoilei	+ +	+ +	+	+ +	–
RD61	+ +	+ +	+	+ + +	–

reindeer (GenBank Accession No. U16369; RD1, GenBank Accession No. AF158711, respectively). An alignment of these sequences was derived from the BLAST search and from manual alignment of the hypervariable regions (Fig. 2).

The BLAST search showed the RD63 parasite SSU rRNA gene sequence was most similar to that of *B. bigemina* type C SSU rRNA (EMBL No. X59607.1). High similarity to portions of the *B. odocoilei,* RD1, and *B. divergens* SSU rRNA genes was also shown by the BLAST search. However, alignment of the entire RD63 gene was easily accomplished only with the *B. bigemina* gene (Fig. 3). Similar alignment could not be done with *B. odocoilei,* RD1, and *B. divergens* SSU rRNA genes because numerous gaps and insertions resulted.

Table 4 gives relative percentage differences determined from the alignment of RD61, RD1, and *B. odocoilei* SSU rRNA gene sequences in Fig. 2, and from the alignment of RD63 and *B. bigemia* in Fig. 3. Corresponding comparable values could not be determined among the RD63, RD61, RD1, and *B. odocoilei* SSU rRNA genes because of the gaps and insertions that resulted from the alignment.

The nucleotide sequences for the RD61 and RD63 SSU rRNA genes were submitted to the GenBank database and assigned accession numbers AF411337 and AF411338, respectively.

Discussion

Two morphologically and genetically distinct *Babesia* spp. were isolated from reindeer located in Placer County, Calif. At present, the species designations for these reindeer piroplasms are unclear. Traditionally, *Babesia* species have been delineated based on such biological characteristics as morphology, the mammalian host, tick vector, geographic origin, clinical disease, antigenic characteristics determined by serological methods, and ultrastructural features. At this time, neither the host range nor the tick vector(s) for the California reindeer *Babesia* spp. are known. The identification of the *Babesia* spp. found in the California reindeer is also hampered by a paucity of reports of *Babesia* spp. infecting deer in the United States. *Babesia odocoilei* has been reported in white-tailed deer and other deer hosts from New Mexico, Texas, Virginia, and Minnesota (Spindler et al. 1958; Robinson et al. 1968; Perry et al. 1985; Holman et al. 2000). A *Babesia* sp. isolate, RD1, identified in a reindeer residing in the same area of California as the reindeer in the current study (Kjemtrup et al. 2000), is very similar in SSU rDNA sequence to *B. odocoilei* (Fig. 2). Aside from *B. odocoilei* and RD1, the only reported *Babesia* sp. found in deer in the United States is a large babesia that was isolated from mule deer (*Odocoileus hemionus)* in California (Thomford et al. 1993).

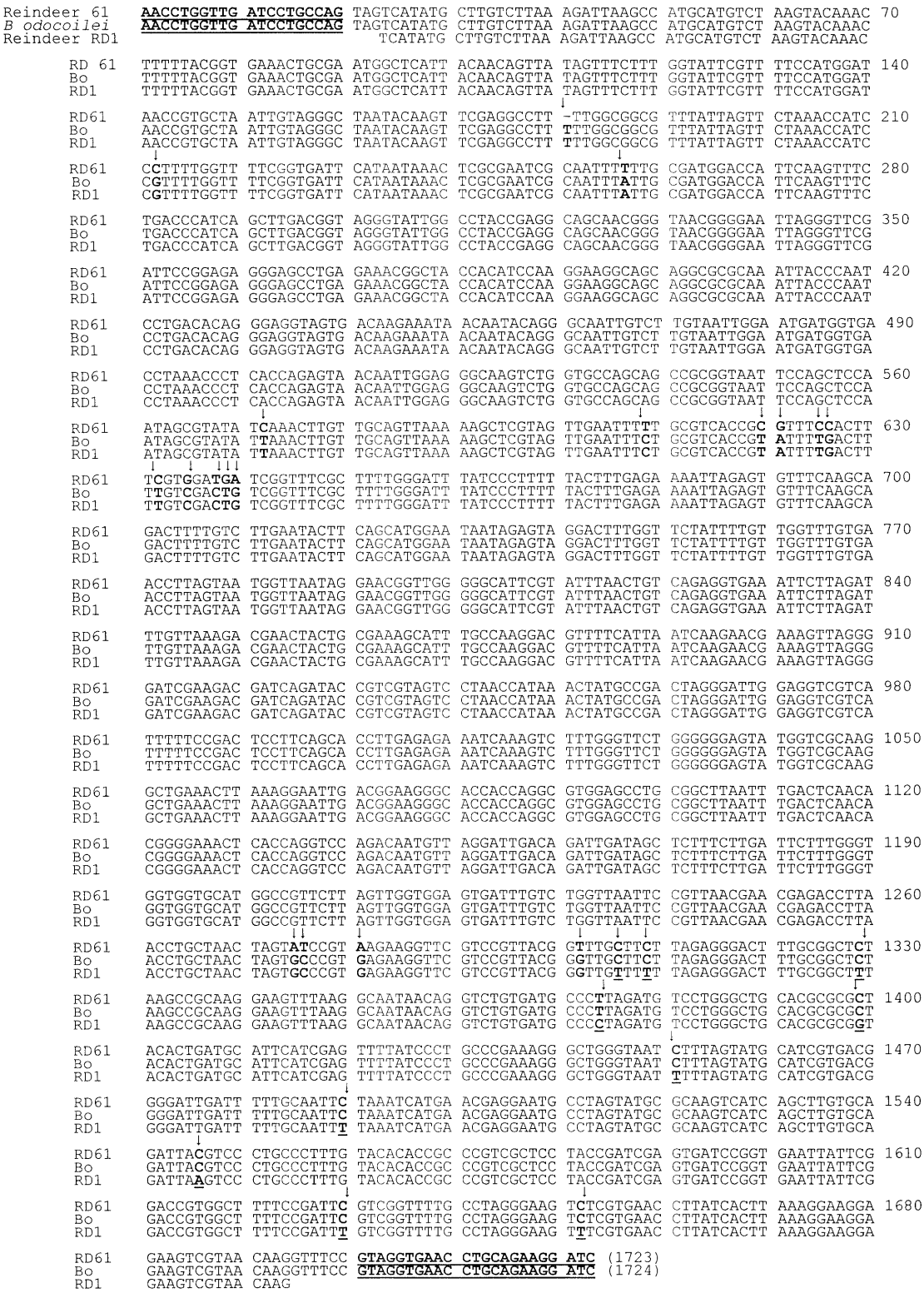
Serum samples from the reindeer in the current study reacted with *B. odocoilei* parasites in the IFA test. In fact, serum from all three infected reindeer reacted equally strongly with both the RD61 reindeer isolate and *B. odocoilei* antigen in the assay, regardless of whether they were infected with the smaller piroplasm or the larger one. These results may indicate one or more possiblities: (1) that the RD61 babesia isolate is *B. odocoilei*, (2) that all three reindeer were exposed to both *B. odocoilei* and the RD61 reindeer isolate, or (3) that both reindeer isolates cross-react strongly with heterologous *Babesia* spp. antigens.

Molecular characterization based on sequence analysis of SSU rDNA or other genetic markers has added a new dimension in defining *Babesia* spp. isolates. The RD1 *Babesia* sp. isolate identified in a reindeer differs at only seven SSU rDNA nucleotide positions from that of *B. odocoilei* (Fig. 2). *B. odocoilei* was originally de-

Fig. 2 Alignment of RD61, *Babesia odocoilei*, and reindeer babesia isolate RD1 (GenBank accession nos. AF411337, U16369, and AF158711, respectively) SSU rRNA gene sequences. Nucleotide differences among the sequences are in *bold type* and *flagged.* Nucleotides in the RD1 sequence that differ from both RD61 and *B. odocoilei* are *underlined.* The oligonucleotide primer sequences used to amplify the RD61 and *B. odocoilei* genes are in *bold type* and underlined. Nucleotide positions are indicated to the right of the alignment; the gene sizes for *B. odocoilei* and RD61 are indicated in parentheses at the end of the alignment

scribed as a parasite of white-tailed deer (*Odocoileus virginianus*), but was later confirmed by SSU rRNA gene-sequence analysis as the causative agent of babesiosis in a caribou in Minnesota (Petrini et al. 1995; Holman et al. 2000) and also as an infectious agent of elk (Holman et al. 2000).

In the current study, the SSU rDNA of the smaller piroplasm, RD61, differs from *B. odocoilei* SSU rDNA in 18 positions and from that of RD1 in 28 positions (Fig. 2). At present there is no consensus as to the degree of identity two SSU rDNA sequences must share in order to be considered the same species. Two distinct



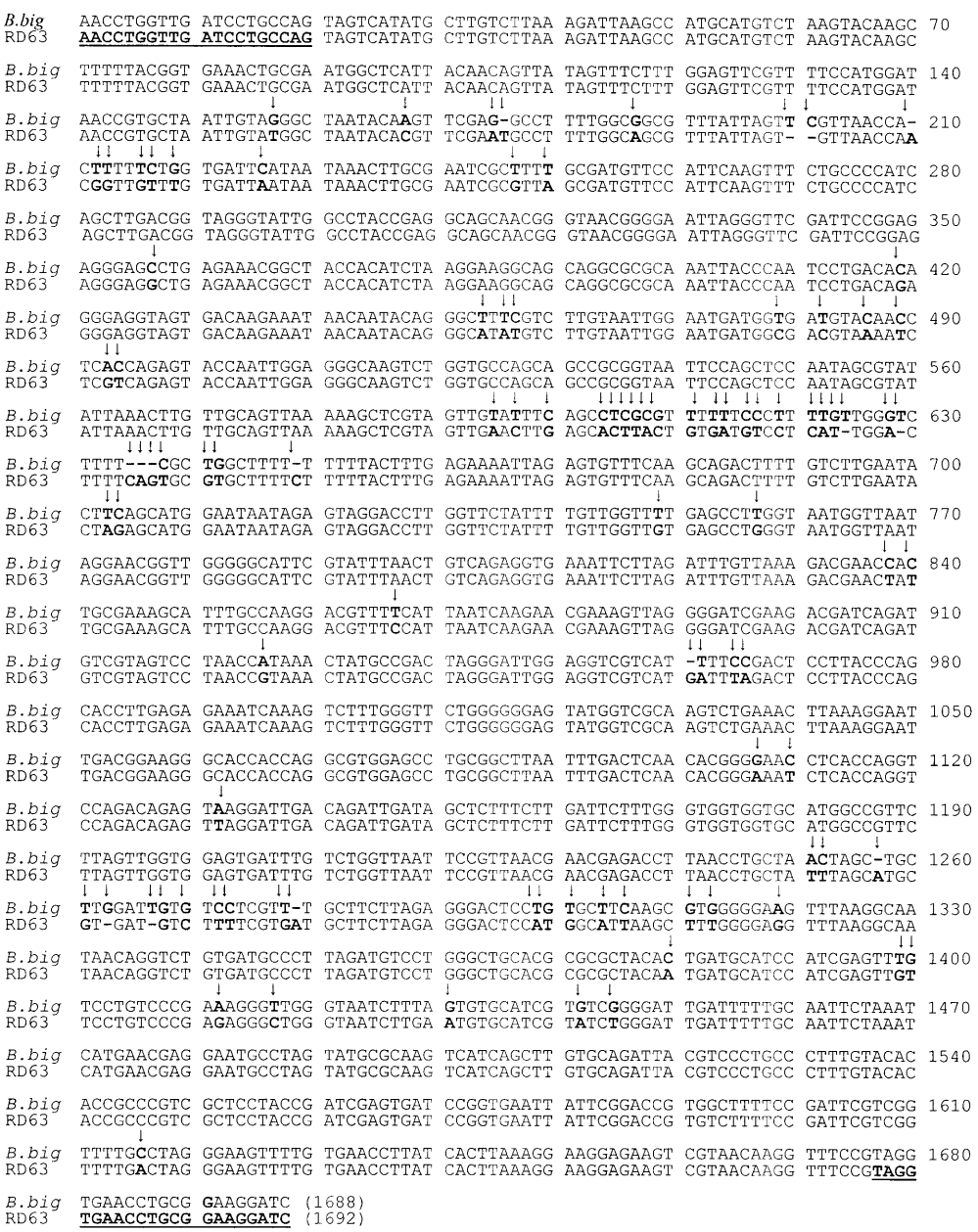


Fig. 3 Alignment of RD63 and *B. bigemina* gene C (GenBank accession no. AF411338 and EMBL no. X59607.1, respectively) SSU rRNA gene sequences. Differences in nucleotide positions are in *bold type* and *flagged*. The oligonucleotide primer sequences used to amplify the RD63 gene are in *bold type* and underlined.. Nucleotide positions are indicated to the right of the alignment; the gene size for each organism is indicated in parentheses at the end of the alignment

Table 4 Percent identity between SSU rRNA gene sequences of various *Babesia* spp. *ND* Not done

	RD61	*B. odocoilei*	*B. divergens*	RD63
RD1	98.4	99.4	97.8	ND
B. odocoilei	99.0	100	98.2	ND
B. divergens	98.3	98.2	100	ND
B. bigemina	ND	ND	ND	94.2

species, *B. divergens* and *B. odocoilei*, differ at only 32 positions (Holman et al. 2000). Clearly, further studies are needed to unequivocally determine if these reindeer parasites are *B. odocoilei* isolates.

The SSU rRNA gene-sequence analysis clearly shows the RD63 babesia isolate to be distinct from other large piroplasms for which SSU rRNA gene-sequence data is available (Fig. 3). Although the RD63 SSU rRNA gene shows most homology with that of *B. bigemina,* (C type gene), there are 87 nucleotide differences between them, discounting differences in the primer regions that may vary depending on the primers used to amplify the gene (Fig. 3). Furthermore, *B. bigemina* is a parasite of cattle and was eliminated from the United States in the late 1930s as a result of an extensive campaign to eradicate the vector tick, *Boophilus* spp. Therefore it is highly unlikely that *B. bigemina* occurs in this area of California.

Babesia spp. morphologically similar to the isolates from the California reindeer have been described in reindeer in Europe. Firstly, *Babesia jakimovi*, a large piroplasm, has been described in Siberian reindeer (Nikol'skii et al. 1977) and is consistent in morphology to

the RD63 piroplasm found in reindeer 62 and 63. Since there is no SSU rDNA sequence data available for *B. jakimovi,* the possibility that RD63 is, in fact, *B. jakimovi* cannot be ruled out. Secondly, experimental infection of reindeer with *B. divergens,* a parasite consistent in size with the RD61 isolate, has been reported (Nilsson et al. 1965). We can, on the basis of the differences in SSU rRNA gene sequences between RD61 and *B. divergens,* rule out *B. divergens* as the identity of the RD61 isolate. However, the possibility exists that the RD61 *Babesia* sp. may be the same as the earlier described organisms, *Francaiella tarandi rangifer* (Yakimoff and Kolmakoff 1929) or *Piroplasma tarandi rangifer* (Chambers 1921).

Although specific epithets for these reindeer babesia isolates could not be assigned by this study, the SSU rRNA gene sequences and morphological descriptions of these parasites will provide a basis for comparison with other piroplasms from cervine hosts as they are described.

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