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IN VITRO ISOLATION AND CULTIVATION OF A *BABESIA* FROM AN AMERICAN WOODLAND CARIBOU (*RANGIFER TARANDUS CARIBOU*)

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ABSTRACT: A *Babesia* species isolated from a captive caribou (*Rangifer tarandus caribou*) with clinical signs of babesiosis and a circulating parasitemia was cultured in vitro. Normal adult caribou erythrocytes supported the growth of the *Babesia* sp., as did erythrocytes from white-tailed deer (*Odocoileus virginianus*). Two basal media (M-199 and RPMI-1640) and a defined medium (HL-1) each supplemented with adult bovine serum were compared. The most favorable growth of the parasite occurred in HL-1 medium with 20% adult bovine serum. The morphology of this *Babesia* sp. isolate shared some characteristics with *B. odocoilei* and *B. divergens*.

Key words: *Babesia* sp., caribou, *Rangifer tarandus caribou*, babesiosis, zoo.

INTRODUCTION

Babesia odocoilei and a large *Babesia* parasite, both found in white-tailed deer (*Odocoileus virginianus*), and recent *Babesia* sp. isolates from mule deer (*Odocoileus hemionus*) constitute the reported *Babesia* spp. of North American cervids (Spindler et al., 1958; Emerson and Wright, 1968, 1970; Thomford et al., 1993). The potential impact of reservoir infection is of importance to the growing industry of exotic hoofstock ranching and farming and to other handlers of exotic or native wildlife, including zoological parks. We describe the in vitro culture of a *Babesia* sp. from a zoo-housed American woodland caribou (*Rangifer tarandus caribou*) with a naturally acquired case of fatal acute babesiosis.

In vitro cultivation has proven to be a valuable tool in the study of *Babesia* spp. (Winger et al., 1987; Goff et al., 1988). Cultured *B. odocoilei* (Holman et al., 1988) from white-tailed deer has been used in infectivity, vector transmission, and epidemiological studies (Waldrup et al., 1989, 1992; Waldrup, 1991). Culture may provide a means of obtaining needed quantities to aid in classification, especially in cases where isolates from heretofore unreported hosts are similar morphologically and require additional means of identification. We report the isolation through culture and the culture maintenance of a *Babesia* sp. isolated from a naturally infected North American caribou (*Rangifer tarandus caribou*) in the Minnesota Zoological Garden, Apple Valley, Minnesota (USA). The morphological relationship to two other cultured *Babesia* species known to infect cervids also is presented.

MATERIALS AND METHODS

A 3-yr-old captive-born and reared male caribou (Minnesota Zoo accession 5750) at the Minnesota Zoological Garden was examined to investigate the cause of hemoglobinuria. The animal was immobilized on 8 November 1991 with 600 mg ketamine hydrochloride (Ketaset, Fort Dodge Laboratories, Fort Dodge, Iowa, USA) and 120 mg of xylazine (Anased, Lloyd Laboratories, Shenandoah, Iowa) delivered by a capture dart (Cap-Chur Equipment, Palmer Chemical and Equipment, Douglasville, Georgia, USA). Blood was collected from the cephalic vein and transferred aseptically to tubes containing ethylenediamine tetraacetic acid (EDTA), 15 mM final concentration. Based on Giemsa-stained blood films, we observed numerous *Babesia* sp. organisms. The blood was refrigerated and later shipped on ice to Texas A&M University (TAMU), College Station, Texas (USA), arriving on 2 December 1991. Blood samples also were drawn from two similarly sedated asymptomatic caribou (Minnesota Zoo accession 6555 and 6536) on 22 November 1991, and the samples immediately sent on ice to TAMU.

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Uninfected erythrocytes (RBC's) for use in cultures of the caribou *Babesia* sp. isolate were prepared from blood collected in EDTA from caribou 6555 and 6536 and from an adult white-tailed deer (kindly provided by Drs. Duane Kraemer and Mark Westhusin, Department of Veterinary Physiology and Pharmacology, Texas A&M University, College Station, Texas), and from defibrinated blood from a normal adult donor steer. Blood collected in EDTA was centrifuged at 500 × *g* and the plasma removed and discarded. The cell pellet was washed three times by centrifugation at 500 × *g* for 15 min in 10 to 15 volumes of 0.15 M phosphate buffered saline with 15 mM EDTA, with removal of the buffy coat at each wash. After the final wash, the supernatant and the top one-third of the cell pellet were removed and the remaining cell pellet was resuspended in an equal volume of Puck's saline glucose (Puck et al., 1958) (Gibco, Grand Island, New York, USA) with 20 g/l glucose added (PSG+G) and stored at 4 C until use. Defibrinated bovine blood was centrifuged as above and the serum decanted and saved for use in medium. The top one-third of the cell pellet was removed and discarded. The remaining RBC's were resuspended in an equal volume of PSG+G and stored at 4 C until use.

Blood samples from infected caribou 5750 and the two asymptomatic caribou, 6555 and 6536, were prepared for culture conditions as follows. Each sample was centrifuged to obtain a cell pellet and the plasma was removed and discarded. The cell pellet was washed three times by centrifugation, but without removal of the buffy layer. After the last wash, the supernatant was removed. The samples from the asymptomatic caribou were dispensed in 0.2 ml aliquots from the packed cell pellets (gently mixed by stirring) to duplicate wells of a 24-well culture plate. One ml of medium was added to each well. The RBC's were incubated at 37 C in a humidified atmosphere of 2% oxygen, 5% carbon dioxide (CO_2), and 93% nitrogen. The wells were fed daily by removing 1 ml of medium overlying the cell layer and replacing with 1 ml appropriate fresh medium. The cultures were maintained for 1 mo with the addition of 50 µl appropriate RBC suspension every 5 to 7 days. Because of the high *Babesia* sp. parasitemia and the poor condition of the erythrocytes, the washed RBC's from infected caribou 5750 were mixed with uninfected RBC's at culture initiation as follows. The final pellet of 5750 infected blood was resuspended in an equal volume of PSG+G to a volume of 0.6 ml. Wells received 0.1 ml 5750 infected RBC suspension, 0.1 ml caribou 6555 RBC suspension or 0.1 ml normal adult bovine RBC suspension, and 1.0 ml of medium. Three media were used, resulting in six different cultures (Table 1). The media included RPMI-1640 (JRH Biosciences, Lenexa, Kansas, USA) buffered with 20 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) (Sigma Chemical Company, St. Louis, Missouri, USA) and M-199 (JRH Biosciences) buffered with 20 mM N-tris(hydroxymethyl)methyl-2-aminoethane-sulfonic acid (TES) (Sigma Chemical Co.), each supplemented with 40% adult bovine serum and 2 mM l-glutamine (JRH Biosciences). A defined medium, HL-1 (Hycor Biomedical Inc., Portland, Maine, USA), also was used. HL-1 medium was supplemented with 20% adult bovine serum and 2 mM l-glutamine. To each medium were added 100 µg/ml streptomycin, 100 U/ml penicillin, 25 µg/ml Fungizone (antibiotic/antimycotic, Gibco) and 100 µg/ml gentamicin (Schering Corporation, Kenilworth, New Jersey, USA). The cultures were incubated and fed appropriate medium daily as above. Subcultures were made every 2 to 4 days depending on growth, using the methods of Holman et al. (1988). At the fifth passage the *Babesia* sp. cultures were transferred to a humidified 5% CO_2 in air atmosphere. Giemsa-stained blood films were made from all caribou samples prior to preparation for culture and from the wells throughout the culture period to monitor parasite growth.

At passages 2, 4, 6, 9 and 11 cultured *Babesia* sp. from 5750 were cryopreserved and stored in liquid nitrogen (−196 C) as described by Holman et al. (1988) except that 20% polyvinylpyrrolidone-40 (PVP) (Sigma Chemical Company) served as the cryoprotectant. Cultured parasites cryopreserved in the sixth passage were reestablished in vitro 1 mo later by rapid thawing in a 37 C waterbath and immediately placing 0.25 ml in a well containing 0.9 ml HL-1 medium and 0.1 ml normal caribou RBC suspension. The well was fed new medium daily. On days 3 and 5 after recovery, 50 µl of normal caribou RBC suspension was added to the well after feeding. On days 7 and 9 subcultures were made. The cultures were cryopreserved on day 12.

At the 13th passage, caribou *Babesia* sp. cultured in caribou RBC's were passed into wells containing RBC's from a white-tailed deer and HL-1 medium supplemented with 20% white-tailed deer serum. The cultures were continually subcultured. Cultures were cryopreserved after four subcultures and a sample reestablished in vitro 1 wk later as above, except donor white-tailed deer RBC's replaced the caribou RBC's. Media with normal adult bovine or deer serum were compared for growth support upon retrieval from liquid nitrogen storage. The caribou *Babesia* sp. cultured in caribou RBC's and cryopreserved at passage 11 was reestablished

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TABLE 1. Percent parasitemias observed in the two types of erythrocytes and three media used to initiate caribou *Babesia* sp. cultures, through day 8. Percent parasitemias are based on 1,000 erythrocytes.

	Caribou erythrocytes			Bovine erythrocytes		
Day	HL-1*	M-199	RPMI-1640	HL-1	M-199	RPMI-1640
1	2.3	2.4	2.2	2.2	1.9	3.1
2	2.4	1.9	1.8	1.4	0.4	1.3
3	2.4	2.5	3.9	0.6	1.6	2.3
4	4.6	2.0	1.9	0.7	0.9	0.9
5	7.8	0.4	ND*	ND	0.3	ND
6	9.1	0.3	0.2	0.1	0.1	0.1
7	7.5	0.6	End	End	End	End
8	7.5	End				

* ND, not done.

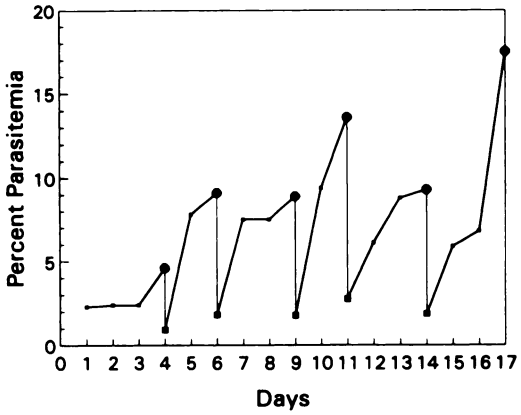


FIGURE 1. Caribou *Babesia* sp. isolate proliferation in caribou erythrocytes from culture initiation through the sixth passage. Percent parasitemia at subculture (●) and the calculated percent parasitemia immediately after subculture (■) are indicated.

20 mo later in white-tailed deer RBC's and medium containing normal adult bovine serum.

Two *Babesia* spp. known to infect cervids were cultured for morphological comparison to the caribou *Babesia* sp. isolate. *Babesia divergens* cultures in bovine erythrocytes were established from infected gerbil (*Meriones unguiculatus*) blood (kindly provided by Dr. J. S. Gray, Department of Agricultural Zoology and Genetics, Faculty of Agriculture, University College–Dublin, Dublin, Republic of Ireland) using methods described for *B. odocoilei* cultures by Holman et al. (1988). The *B. odocoilei* used was a previously described Texas isolate (Holman et al., 1988) and was cultured in white-tailed deer RBC's.

RESULTS

Based on the Giemsa-stained thin smears of caribou 5750 blood made upon arrival at TAMU, there were numerous intracellular and extracellular *Babesia* sp. parasites. No parasites were observed in Giemsa-stained blood films from the two asymptomatic caribou, 6555 and 6536.

Cultures from caribou 5750 infected blood contained healthy appearing *Babesia* sp. under all culture conditions for the first 3 days after establishment (Table 1). All cultures except the RPMI-1640 and HL-1 with bovine RBC's were passed on day 4. The RPMI-1640 with bovine RBC's culture was passed on day 5. On day 6 a few healthy parasites could still be found in the HL-1 with bovine RBC's culture, and 50 μl of bovine RBC suspension was added in an attempt to help the growth of the parasites. However, due to declining percent parasitemias and the gradual disappearance of parasites from all cultures except the HL-1 with caribou RBC's, only the HL-1 with caribou RBC's culture was continued beyond day 7 (Table 1). The HL-1 with caribou RBC's culture survived the first passage and continued to thrive. The maximum parasitemia observed was 18% (Fig. 1). Cryopreserved sixth passage parasites were easily reestablished in caribou RBC's as evidenced by a successful passage 7 days after revival (Fig. 2).

The caribou *Babesia* sp. grew readily in white-tailed deer erythrocytes (Fig. 3). Cryopreservation and subsequent reestablishment of the parasites in white-tailed deer RBC's was successful in medium supplemented with either deer or bovine serum (Fig. 4). Cryopreserved parasites in caribou RBC's also were successfully established in white-tailed deer RBC's upon recovery from liquid nitrogen (data not shown).

The caribou *Babesia* sp. isolate, *B. divergens* and *B. odocoilei* frequently were observed as similar small pyriform, flattened or oval parasites, often paired, located at the periphery of the cell in close proximity

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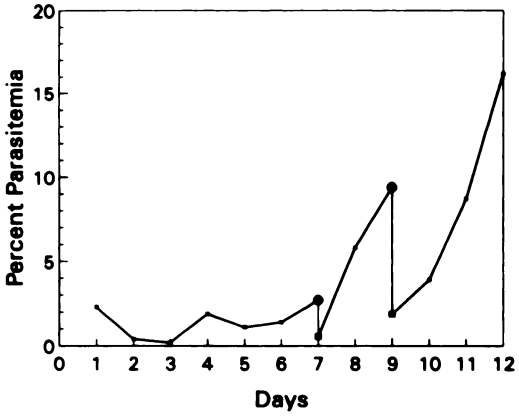


FIGURE 2. Reestablishment of cryopreserved caribou *Babesia* sp. in caribou erythrocytes and medium supplemented with bovine serum. Percent parasitemia at subculture (●) and the calculated percent parasitemia immediately after subculture (■) are indicated.

to the limiting membrane of the host erythrocyte. The caribou *Babesia* sp. isolate cultured in caribou RBC's was characterized by small pyriforms and ring forms and by large pleomorphic forms, particularly along the edge of the cell (Fig. 5). In early passages numerous large thready forms and forms that encircled the inside periphery of the host erythrocyte were seen. These forms were less prominent in later passages, but were frequently

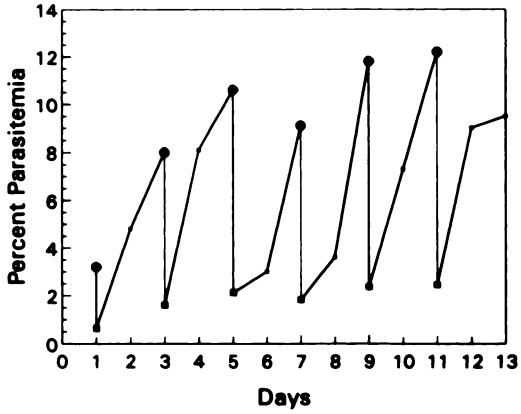


FIGURE 3. Typical growth of the caribou *Babesia* sp. in white-tailed deer erythrocytes and deer serum-supplemented medium, passages 14 through 18. Percent parasitemia at subculture (●) and the calculated percent parasitemia immediately after culture (■) are indicated.

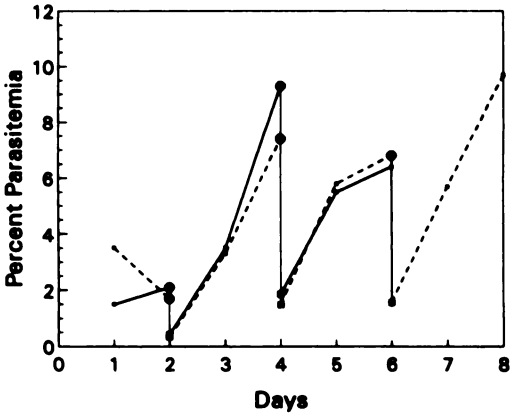


FIGURE 4. Reestablishment of cryopreserved caribou *Babesia* in white-tailed deer erythrocytes. Growth in medium supplemented with deer serum is shown by the solid line and growth in medium supplemented with bovine serum is shown by the broken line. Percent parasitemia at the time of subculture is shown by ● and the calculated percent parasitemia immediately after subculture is shown by ■.

seen upon reestablishment of cryopreserved cultures. Individual caribou *Babesia* sp. pyriforms closely resembled pyriforms of *B. odocoilei* (Fig. 6), but the large peripheral and thready forms characteristic of the caribou isolate (Fig. 5) were not seen in *B. odocoilei*. The organization of the organisms in multipli-parasitized erythrocytes was distinctive in that *B. odocoilei* tended to be crowded in the cell with no apparent order, but the caribou isolate tended to be more neatly arrayed, frequently in a circular arrangement. Cells containing more than four parasites commonly were seen with both the caribou isolate and *B. odocoilei*, but were not observed with *B. divergens* cultured in bovine erythrocytes (Fig. 7). *Babesia divergens* occurred predominantly as single organisms, although paired forms and occasional tetrads were also observed.

No *Babesia* sp. were detected in cultured erythrocytes from either of the asymptomatic caribou during a month of maintenance.

DISCUSSION

The caribou *Babesia* sp. isolate was readily adapted to in vitro cultivation in

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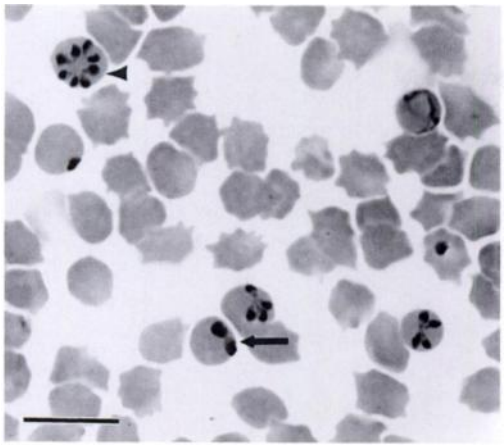


FIGURE 5. Cultured caribou *Babesia* sp. in caribou erythrocytes. Included are large pleomorphic forms, edge forms, and small pyriforms. An erythrocyte with numerous parasites uniformly arrayed is shown (arrowhead). Paired parasites located at the erythrocyte margin are indicated by the arrow. Giemsa. Bar = 10 μm.

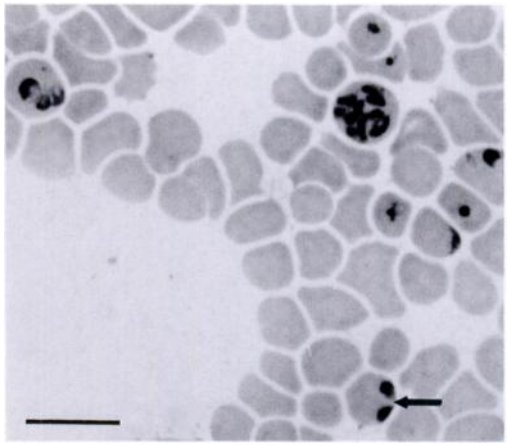


FIGURE 6. Cultured *Babesia odocoilei* in white-tailed deer erythrocytes. Numerous parasites within an erythrocyte are shown. Paired pyriforms located at the erythrocyte margin are indicated by the arrow. Giemsa. Bar = 10 μm.

both caribou erythrocytes and white-tailed deer erythrocytes and demonstrated a lack of fastidiousness in serum requirements. The parasite proliferated rapidly and it was necessary to adhere to a 2-day subculture schedule to maintain healthy appearing parasites. The ease of culture establishment and reestablishment after cryopreservation was similar to that reported for *B. odocoilei*. The caribou isolate achieved high parasitemias more quickly after culture initiation and could be subcultured earlier than *B. odocoilei* (Holman et al., 1988), but this may be attributed to several factors. The infected caribou blood used for culture initiation was highly parasitemic and the *B. odocoilei* infected deer blood was not; the defined medium used in the caribou *Babesia* sp. cultures may have contributed to more vigorous growth; or inherent growth differences may exist between the two isolates.

We substituted white-tailed deer RBC's for caribou RBC's in the culture system because deer blood was more readily available. The ease of parasite adaptation to deer erythrocytes and the successful reestablishment in deer RBC's after cryopreservation make this system a viable alternative to culturing in caribou RBC's. This culture method may be useful for characterization of new *Babesia* sp. isolates found in other native or exotic wildlife ruminants if it is difficult to acquire a continuous source of erythrocytes from the same host species.

The ready utilization of white-tailed deer erythrocytes and medium containing deer serum may indicate a close relation-

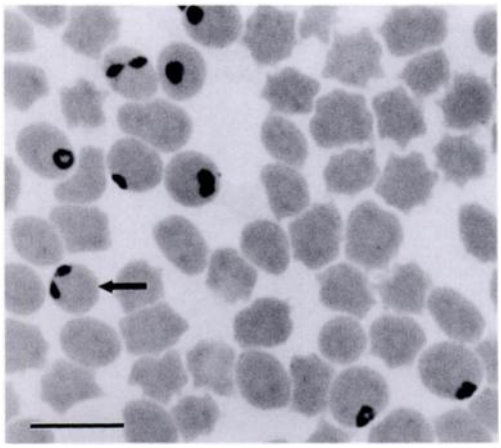


FIGURE 7. Cultured *Babesia divergens* in bovine erythrocytes. Single and paired organisms are shown. Paired pyriforms located at the erythrocyte margin are indicated by the arrow. Giemsa. Bar = 10 μm.

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ship of this particular isolate to *B. odocoilei*. Our data were insufficient to determine unequivocally if the isolate is, in fact, a strain of *B. odocoilei*. If so, there are considerable etiological implications since the distribution of both white-tailed deer and known vector ticks for *B. odocoilei* transmission is widespread. The potential for dissemination of the disease is great, although our finding was an isolated case. Tick and disease control regimens may become increasingly important as captive wildlife ruminants experience stress and increased disease susceptibility despite efforts to create stress-free environments. Studies are presently underway to clarify the relationship between this isolate and other *Babesia* species with similar morphology, especially those reported from other wildlife host species.

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