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In vitro cultivation of a newly recognized *Babesia* sp. in dogs in North Carolina

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Abstract

A novel large *Babesia* sp. from an infected dog was cultivated in vitro by microaerophilous stationary phase culture methodology. A primary culture initiated in enriched RPMI-1640 medium supplemented with 40% canine serum and incubated in a 2% oxygen environment supported parasite growth in vitro. Subsequent subcultures into enriched HL-1 medium with 20% fetal bovine serum also supported parasite propagation. Cultures were successfully introduced to 5% carbon dioxide in air atmosphere at passage 4. To date, the parasites have been continuously cultured through 35 passages, although the parasitemias are low, ranging from 0.2 to 0.3%. Parasites cultured in RPMI with canine serum were cryopreserved and successfully recovered from liquid nitrogen storage. The small subunit ribosomal rRNA gene sequence was identical in blood-derived and culture-derived parasites, differing in a single base position from the previously reported sequence for this *Babesia* sp. The ultrastructure of the parasite was consistent with that of other large *Babesia* spp., except that the spherical body contained numerous round particles unlike the inclusions previously described in *Babesia* spp.

Keywords: *Babesia* sp.; Canine babesiosis; HL-1 medium; Microaerophilous stationary phase culture; RPMI-1640; Small subunit ribosomal RNA gene; Transmission electron microscopy

1. Introduction

Canine babesiosis is most commonly caused by the tickborne hemoprotozoans *Babesia gibsoni*, a small piroplasm, and *Babesia canis*, a large piroplasm, which have been recognized since the early 1900s (Piana and Galli-Valerio, 1895; Patton, 1910). Recently, two additional canine *Babesia* spp. have been reported in the United States; *Babesia conradae*, a small piroplasm, in California and an unnamed large piroplasm in North Carolina (Conrad et al., 1991; Birkenheuer et al., 2004; Kjemtrup et al., 2006). The latter, first described in a Labrador retriever undergoing chemotherapy for lymphoma, is similar in morphology and size to *B. canis*. However, this large piroplasm is genetically distinct from *B. canis* based on small subunit ribosomal rRNA (SSU rRNA) gene sequence analysis, sharing the highest degree of sequence identity (greater than 93%) with the large piroplasms *Babesia bigemina* of

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cattle and *Babesia caballi* of horses. This novel canine *Babesia* sp. shares lesser identity (less than 92%) with the corresponding sequences of the three *B. canis* subspecies, *B. c. canis*, *B. c. vogeli*, and *B. c. rossi*. Phylogenetic analysis places the canine *Babesia* sp. in the *Babesia* spp. *sensu stricto* clade with good statistical support (Birkenheuer et al., 2004).

When this *Babesia* sp. was first reported, it was unknown whether the parasite was a new species of *Babesia*, or if it was previously described with no reported genetic data (Birkenheuer et al., 2004). The clinical signs and hematological parameters of the infected dog were consistent with those of babesiosis. However, the dog was immunosuppressed at the time of parasitemia, suggesting that the parasite may not be host-specific to the canine and that babesiosis ensued as a direct consequence of the compromised immune state (Birkenheuer et al., 2004). However, since that time an additional five cases of babesiosis due to this parasite have been diagnosed in North Carolina dogs by the Tickborne Diagnostic Laboratory (Department of Clinical Sciences, North Carolina State University, Raleigh, NC; unpublished results), suggesting that dogs are a natural host for this parasite. Since the complete life cycle of this parasite is not elucidated, the tick vector(s) and reservoir host(s) remain unknown.

Characterization of numerous *Babesia* species has been facilitated by establishing in vitro cultures of the parasites (Droleskey et al., 1993; Holman et al., 1994b, 2005; Schetters et al., 1997). Cultures provide a source of parasites for in vivo inoculations to establish host range, in vitro determination of erythrocyte specificity, comparative morphology, and for vaccine and diagnostic test development (Moreau et al., 1988; Montenegro-James et al., 1989; Spencer et al., 2006). To date, a number of *Babesia* species, including the two common canine parasites, *B. canis* and *B. gibsoni*, have been successfully cultured in vitro using methodology based on the microaerophilous stationary phase (MASP) system pioneered by Levy and Ristic (1980).

The culture establishment, cultured parasite morphology and ultrastructure, and the SSU rRNA gene sequence from this isolate of the large unnamed canine *Babesia* sp. identified in North Carolina dogs are described herein.

2. Materials and methods

2.1. Babesia sp. isolate

Blood collected into ethylenediaminetetraacetic acid (EDTA) from a 12-year-old spayed female German Shepherd dog was submitted to the Tickborne Diagnostic Laboratory at North Carolina State University, Raleigh, NC for diagnostic testing for *Babesia* infection. Large piroplasms were noted during microscopic examination of a thin, stained blood smear. Blood was subsequently sent on ice by overnight shipment to the Department of Veterinary Pathobiology, Texas A&M University, College Station, TX.

2.2. In vitro culture

Red blood cells (RBCs) from the infected blood sample were prepared for culture as follows. The blood was centrifuged at 500 × *g* for 20 min to pellet the cells, and the plasma and buffy layer were removed and discarded. A 0.2 ml aliquot of packed RBC was mixed with 1 ml of 0.15 M phosphate buffered saline (PBS) containing 15 mM EDTA and washed by centrifugation at 885 × *g* for 3 min. The supernatant and buffy layer were removed, and the cell pellet washed two more times as above in 1 ml of PBS. After the final wash, the supernatant was removed and the RBC used as packed cells. The cultures were initiated in duplicate wells of a 48-well culture plate with 80 μl washed packed infected RBC and 720 μl of either RPMI-1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 40% canine serum (Pel-Freez Biologicals, Rogers, AZ), 10 μg/ml final concentration Albumax I (Invitrogen, Carlsbad, CA), and buffered with 20 mM HEPES (R40), or RPMI-1640 medium with 40% canine serum as above, but buffered with both HEPES and 20 mM TES (R40TH) (Table 1). Antibiotics were added to all media at a final concentration of 200 μg/ml streptomycin, 200 U/ml penicillin, and 50 μg/ml Fungizone (Antibiotic–Antimycotic; Gibco BRL, Grand Island, NY). The cultures were incubated at 37 °C in a humidified modular incubator chamber (Billups-Rothenberg Inc., Del Mar, CA) in a gas mixture of 2% oxygen, 5% carbon dioxide, and 93% nitrogen. At passage 4, cultures in R40 medium were introduced to a 5% carbon dioxide in air atmosphere in parallel with cultures maintained in the gas mixture.

Cultures were fed daily by removing 700–720 μl without disturbing the settled RBC layer, and replacing this with 700–720 μl fresh medium, as appropriate. Non-infected donor packed RBC (25 μl), prepared as described below, were added every 7 days. At subculture, the culture medium was replenished as above and RBC resuspended in the fresh medium. Subcultures were performed at a split ratio of 1:4, with transfer of 200 μl of the RBC suspension to a new well. The volumes were brought to 800 μl with fresh medium