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.

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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...
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 anduffy 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...
and 10% donor RBC (v/v) prepared as below. The cultured parasites were subcultured into several media to determine the optimal formulation to support the in vitro growth of the parasite (Table 1).

Non-infected donor RBC were obtained from a 4-year-old neutered male American Pointer mixed breed dog. The donor has not shown any signs of illness since he was acquired at 6 weeks of age and was negative for the presence of canine Babesia spp. by microscopy and PCR. The blood was drawn via venipuncture into Alsever’s solution and then centrifuged at 500 \( g \) for 20 min to pellet the cells. The plasma and buffy layer were removed and a volume of Alsever’s solution equal to the volume of the RBC pellet was added to the tube. The blood was stored at 4 °C and used within 2 weeks.

At use, the non-infected RBCs were washed by centrifugation as described above.

Parasite growth was monitored by examination of Giemsa-stained (Accustain, Sigma, St. Louis, MO) thin blood films at 500× magnification under oil (Labophot-2 microscope, Nikon) at 2-day intervals until parasites were observed and daily thereafter. Early subcultures were subjectively evaluated based on numbers of parasitized erythrocytes per 500 × field. When at least seven parasitized erythrocytes were observed in several 500 × fields, the cultures were passaged. The percent parasitemia at subculture was calculated by enumeration of 1000 total infected and uninfected RBC on Giemsa-stained smears at 1000 × under oil.

Two methods were tested to enhance the parasitemia of the cultures. One was to continue to replenish the medium for several days beyond the day deemed appropriate for subculture. The second was to replenish the medium on the day deemed appropriate for subculture, and to then resuspend the cultured erythrocytes in the fresh medium and plate 200 \( \mu l \) per well with no addition of donor uninfected RBC, but bringing the volume to 800 \( \mu l \) with fresh medium. This effectively reduced the haematocrit of the culture to approximately 2–2.5%. The ensuing parasitemia was monitored daily as above.

2.3. Small subunit ribosomal RNA (SSU rRNA) gene sequence analysis

Parasite DNA was purified from infected dog blood and from cultured parasites following the Flexi-Gene DNA Kit protocol (Qiagen, Valencia, CA). The SSU rRNA gene was amplified, cloned, and sequenced using previously described methods (Holman et al., 2003). The full SSU rRNA gene was sequenced from four clones from the blood-derived and two clones from the culture-derived parasites and the resulting sequences were aligned and consensus sequences determined using Sequencher 3.11 software (Gene Codes Corporation, Inc., Ann Arbor, MI). The individual consensus sequences for the SSU rRNA gene from blood parasites and from cultured parasites were subjected to BLAST similarity searches (National Center for Biotechnology Information, National Institutes of Health; http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul et al., 1990). The obtained sequence was aligned with the corresponding Babesia sp. SSU rRNA gene sequence in the GenBank database (accession no. AY618928; Birkenheuer et al., 2004) (Clustal W http://www.ebi.ac.uk/clustalw/).

2.4. Transmission electron microscopy (TEM)

Cultured Babesia sp. parasites from passage 7 cultivated in R40 medium were prepared for TEM as previously described (Holman et al., 2005).

Table 1
Media tested, the culture level at which each was introduced, and the outcome for each for the Babesia sp. cultured in humidified 2% oxygen atmosphere at 37 °C

<table>
<thead>
<tr>
<th>Name</th>
<th>Basal medium</th>
<th>Serum</th>
<th>Supplement</th>
<th>Buffer</th>
<th>Culture level</th>
<th>Growth supported</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Albumax</td>
<td>H/Ta</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H/1001</td>
<td>HEPES</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R40</td>
<td>RPMI</td>
<td>40% canineb</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>1°</td>
</tr>
<tr>
<td>R40TH</td>
<td>RPMI</td>
<td>40% caninen</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>1°</td>
</tr>
<tr>
<td>R40Dog</td>
<td>RPMI</td>
<td>40% donor dogc</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>6th passage</td>
</tr>
<tr>
<td>R40FBS</td>
<td>RPMI</td>
<td>40% FBSd</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>5th passage</td>
</tr>
<tr>
<td>R20FBS</td>
<td>RPMI</td>
<td>20% FBS</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>22nd passage</td>
</tr>
<tr>
<td>HL20FBS</td>
<td>HL-1</td>
<td>20% FBS</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>5th passage</td>
</tr>
<tr>
<td>HLHT</td>
<td>HL-1</td>
<td>20% FBS</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>8th passage</td>
</tr>
</tbody>
</table>

a Hypoxanthine/thymidine.

b Pooled canine serum, Pel-Freez Biologicals.
c Dog serum from RBC donor animal.
d Fetal bovine serum, HyClone.
3. Results

3.1. Parasite culture

*忽悠* sp. cultures were initiated in R40 and R40TH (containing the buffer TES in addition to HEPES) media, and incubated in a humidified low oxygen tension atmosphere at 37 °C. Parasites were not seen in culture until 13 days after initiation, when a few parasitized erythrocytes were observed in R40 cultures. Passages 1 and 2 of the R40 cultures were performed on days 20 and 22 as the parasitemia reached approximately 0.15 and 0.19%, respectively. Thereafter, passages were performed at 3–7-day intervals as parasitemias of approximately 0.10–0.18% were achieved (Fig. 1). Despite the low parasitemias, all forms of the parasite were observed, including dividing forms (Fig. 2). No parasites were seen in R40TH medium for 30 days after initiation, at which time the cultures were terminated.

At passage 4, R40 cultures were subcultured into two 48-well plates so that parallel cultures were maintained in the low oxygen atmosphere and introduced to a 5% carbon dioxide in air atmosphere (Table 1). The parasites adapted well to the 5% carbon dioxide in air atmosphere and the cultures were then maintained in this environment. Three subsequent subcultures were performed at 3–7-day intervals as the parasitemias reached 0.12, 0.42, and 0.46%, respectively (Fig. 1). At the eighth passage, parasite cultures were used for TEM or to subculture into HLHT medium (HL-1 medium with fetal bovine serum, Albumax, HB101, hypoxanthine, and thymidine; Table 1). Subsequent passages in HLHT medium (currently in passage 35) were at 2–4-day intervals as parasitemias of approximately 0.2–0.3% were achieved (not shown).

Passage 5 parasites in R40 cultivated in the low oxygen atmosphere (above) were subcultured into HL20FBS medium and transferred to a 5% CO₂ in air atmosphere (Fig. 1). Passages 6–10 were performed at 2–5-day intervals. Increasing parasitemias were obtained as the cultures progressed, from <0.1 at passage 6 to 0.33% at passage 10 (Fig. 1).

Although early subcultures were done at varying time intervals from 2 to 7 days depending on parasite growth, later subcultures were done every 2–4 days. The parasitemias remained low, usually 0.2–0.3% at subculture. Attempts to enhance the percent parasitemia during the later passages by extending the time intervals beyond 4 days between subcultures were not successful. A drop in parasitemia ensued, accompanied with increased extracellular parasites (Fig. 3A) and the appearance of degenerating intra- and extracellular

![Fig. 1. Percent parasitemia at subculture for the large Babesia sp. cultured in R40 medium in 2% oxygen, 5% carbon dioxide, and 93% nitrogen atmosphere (shaded), R40 medium in 5% carbon dioxide in air atmosphere (solid), HL20FBS medium in 5% carbon dioxide in air (filled), and in R40 on recovery from liquid nitrogen cryopreservation (filled).](image)

![Fig. 2. Large unnamed Babesia sp. piroplasms cultured in canine red blood cells. Intraerythrocytic parasites were seen as paired forms (large arrowheads), various single forms (small arrowheads), and dividing forms (arrow) (1000×). Giemsa stain. Scale bar, 10 μm.](image)
parasites. For example, passage 5 culture in HL20FBS medium with a parasitemia of 0.22% on day 3 dropped to 0.19% on day 4 (not shown).

The parasitemia increased when the RBC to medium ratio (v/v) was decreased, concomitant with extending the time interval. A drop from a 10% (conventional) to a 2% (reduced) haematocrit generally resulted in an increased parasitemia ranging from approximately 5–13% in 2–3 days. Although some extracellular and degrading forms were apparent in these cultures, the cultures appeared more robust than those simply maintained without reducing the haematocrit (Fig. 3A and B).

In vitro propagation of this canine Babesia sp. was supported by R40, R20FBS, HL20FBS, and HL20HT media (Table 1). None of the additional media formulations tested for their ability to support in vitro growth of this parasite was successful.

Passage 3 cryopreserved cultured parasites were successfully recovered in R40 medium as shown in Fig. 1.

3.2. SSU rRNA gene analysis

The full SSU rRNA gene was successfully amplified, cloned and sequenced from the infected blood-derived parasite sample and from parasites obtained from culture. Aligning the two obtained consensus sequences revealed that both the blood- and culture-derived parasites shared the same SSU rRNA gene sequence (GenBank accession nos. EU109716 and EU109717, respectively). An alignment of this sequence with the SSU rRNA gene sequence (GenBank accession no. AY618928) from the large unnamed Babesia sp. described by Birkenheuer et al. (2004) shows a single base substitution (C for T) at position 721 in the GenBank sequence. This substitution was found in all six cloned sequences obtained in this study and resulted in 99.9% identity with the reported sequence from the large unnamed Babesia sp.

3.3. TEM

The Babesia sp. piroplasm shown in Fig. 4 is bounded by a single limiting membrane with the inner membrane complex evident at the posterior and anterior ends of the parasite. The double membrane bounded nucleus is large and centrally located. A spherical body is located anterior to the nucleus and is filled with numerous small round particles. Rough endoplasmic reticulum is seen throughout the cytoplasm and acristate mitochondrion-like organelles are located in the anterior end of the organism. Rhoptries, which along with micronemes (not shown) comprise the apical complex of the parasite, are evident at the anterior end of the piroplasm.

4. Discussion

A continuous MASP culture of the novel Babesia sp. first described in a dog by Birkenheuer et al. (2004) was successfully established following primary culture in R40 medium containing a commercial pooled dog serum. Parasites subcultured into HL-1 medium with
fetal bovine serum were continuously cultivated as well, but subcultures into RPMI-1640 medium supplemented with serum from the RBC donor dog or FBS were not successful. For culture initiation, RPMI-1640 was selected as the basal medium based on previous reports of this medium supporting in vitro propagation of *B. canis*, a large piroplasm of dogs (Moreau and Soula, 1979; Molinar et al., 1982; Schetters et al., 1997). This medium also supports cultures of the equine large piroplasm, *B. caballi* (Avarzed et al., 1997), which is phylogenetically closely related to the canine *Babesia* sp. cultured in this study (Birkenheuer et al., 2004).

In early work, *Babesia* spp. cultivation relied on autologous serum and RBC from a non-infected donor animal representative of the vertebrate host for culture initiation (Canning and Winger, 1987). The RPMI complete medium used for our *Babesia* sp. primary culture contained pooled canine serum from a commercial source and supported parasite growth. However, medium supplemented with autologous serum from our RBC donor animal failed to support the parasite. It is well documented that the origin and quality of donor serum and erythrocytes for in vitro culture of *Babesia* spp. are critical to the outcome (Canning and Winger, 1987; Holman et al., 1998, 2005; Sunaga et al., 2002; Zintl et al., 2004). Thus, it is possible that the donor dog used in this study was not an optimal source of serum and erythrocytes, and may explain the failure to obtain higher parasitemias in these cultures.

The failure of the donor dog serum to support these parasites and the inability to compare different commercial canine sera due to cost constraints led to testing FBS as a substitute. Although not reported for canine *Babesia* spp. in vitro cultures, fetal bovine serum has been used in place of normal adult host serum in cell culture medium for a number of other *Babesia* spp. (Thomford et al., 1993; Holman et al., 1994a,b; Zintl et al., 2002; Zweygarth et al., 1995b). In our study, passage 4 parasites were successfully subcultured into FBS supplemented HL-1 medium, which then continued to support parasite propagation. Early passage parasites failed to thrive in RPMI-1640 medium with 40% FBS without HB101 supplement, but parasites at passage 22 adapted well to RPMI-1640 with 20% FBS and HB101 supplement. It is possible that the omission of HB101 from former resulted in the failure of the parasites to thrive. Further studies are needed to determine if HB101 is a critical media component for these parasites.

HL-1 was first used for establishing *B. caballi* continuous cultures (Holman et al., 1993), and has since been used for culturing numerous *Babesia* spp., including *B. gibsoni*, a canine small piroplasm, and *Babesia* spp. of other animal hosts as well as man (Zweygarth et al., 1995a; Zweygarth and Lopez-Rebollar, 2000; Zintl et al., 2002; Holman et al., 2005). In the current study, the canine *Babesia* sp. parasites adapted readily to HL-1 medium with 20% FBS, resulting in shorter passage intervals than cultures in RPMI-1640 with dog serum. Moreover, the parasitemias obtained in HL-1 medium met or exceeded those of RPMI cultures. Due to the small size of the original blood sample available for culture, the usefulness of this medium for initiating cultures of this *Babesia* sp. was not tested.

Among the numerous supplements tested for in vitro cultivation of *Babesia* spp., Albumax and hypoxanthine are beneficial in a number of systems with both introduced as culture supplements for the fastidious hemoparasite, *Theileria (Babesia) equi* (Holman et al., 1994a; Zweygarth et al., 1995a). Albumax was included in the original medium formulation that supported the canine parasite in this study, and, because of this, was
also included in all of the additional media formulations tested. Whether it is a critical component remains to be determined since media without it were not tested.

Hypoxanthine is reported to be critical in cultivation of some Babesia spp., to enhance in vitro parasitemias of others, and to supplement defined or reduced serum media for Babesia spp. (Zweygarth et al., 1995a,b; Jackson et al., 2001; Zweygarth and Lopez-Rebollar, 2000; Neves et al., 2001). Thymidine in conjunction with hypoxanthine is reported in media that support in vitro cultivation of Babesia spp. (Holman et al., 1998, 2005; Spencer et al., 2006). The addition of hypoxanthine and thymidine to HL-1 medium in this study did not appear to be critical to the success of the cultures, since parasites also survived in HL-1 medium lacking these additives. RPMI-1640 based formulations lacking hypoxanthine and thymidine were not tested, so it is unknown if their inclusion affected the outcome of the primary culture.

The canine Babesia sp. in this study adapted to culture and was successfully established, however the percent parasitemias remained low compared to those reported for other Babesia spp. cultures (Holman et al., 1993, 1998). It is interesting that these parasites may be continuously cultured in a 5% carbon dioxide in air atmosphere despite the low parasitemias. Generally, it is reported that this atmosphere will only support Babesia spp. cultures with 1% parasitemias (reviewed by Canning and Winger, 1987). Attempts to boost the parasitemia of the canine Babesia sp. in vitro by extending the intervals between passages resulted in increased numbers of extracellular parasites, which often appeared degraded, and decreased intracellular parasites. As mentioned above, successful Babesia sp. propagation is dependent on the donor RBCs and serum. Thus, the lower parasitemias might also be attributed to a less than optimal donor animal available for this study. Perhaps higher parasitemias might be achieved with a different donor animal. Sera and erythrocytes from other donors should be tested for their ability to support this Babesia sp. in vitro. It is also possible that a vital nutrient was rapidly depleted in these cultures since reducing the haematocrit resulted in higher parasitemias. In this case, alternate media formulations might result in higher parasitemias.

This isolate of the novel canine Babesia sp. has a single base substitution (cytosine for a thymidine) at position 720 in the 18S rRNA gene sequence previously reported for the Babesia sp. isolate (AY618928; Birkenheuer et al., 2004) (Fig. 4). This substitution occurred in both the blood- and culture-derived 18S rRNA gene sequences, and is also present in the gene sequences from two other large Babesia spp., Babesia caballi (GenBank accession no. Z15104) and Babesia bigemina (unpublished data). An alignment of other Babesia spp. 18S rRNA gene sequences from the GenBank database reveals that this position may variably hold either C or T. Since Babesia species possess multiple copies of the ribosomal RNA transcriptional unit (Reddy et al., 1991), it appears that different copies of the canine Babesia sp. SSU rRNA genes may hold different bases in this position.

The ultrastructure of the canine large Babesia sp. is similar to that of the phylogenetically closely related species, B. caballi (Kawai et al., 1999; Birkenheuer et al., 2004). The spherical body in the canine Babesia sp., however, contains numerous small round particles that are surrounded by an electron-dense covering, whereas the B. caballi spherical body is reported to contain electron-dense granules. Further studies are needed to clarify the exact nature of these structures in the two species.

The culture establishment of this Babesia sp. provides a continuous source of parasites for further studies without the need for infecting dogs. The availability of these parasites will allow optimization of culture conditions, production of antigens or other reagents for designing diagnostic tests, production of immunogens for vaccine development, and studies of the biology of these parasites.

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References


