Cultivation of *Babesia* and *Babesia*-Like Blood Parasites: Agents of an Emerging Zoonotic Disease

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**INTRODUCTION**

Members of the genus *Babesia* are better known to those with an interest in animal parasitology than those focused on human disease, but in recent years, there has been a developing interest in this protozoan parasite with recognition of its role as a zoonotic agent of human disease, i.e., a disease communicable from animals to humans. Babesiosis, as the disease is known, is found in a wide variety of mammals but is perhaps most prevalent in rodents, carnivores, and cattle (50). In excess of 100 species of *Babesia* have been described from mammals. Like the malaria agent, the parasite attacks and damages host erythrocytes. The disease is transmitted to humans by infected ticks, a route shared with the agents of spotted fever, borreliosis, and ehrlichiosis; human-to-human transmission does not occur with the exception of transfusion-mediated spread (7, 17). *Babesia* is placed in the same taxonomic group—the Pirolasmas—as another blood parasite, *Theileria*. The group name derives from the pear-shaped appearance of the parasites (pirolasmas) as seen in infected erythrocytes (pirolasmosis). *Theileria*, however, has not been reported from humans. The isolation of a new strain of *Babesia* (WA-1) with features of *Theileria* and the reexamination of several existing babesias as possible theilerias has introduced uncertainty in the separation of the two genera (26, 52).

*Babesia* as Zoonotic Agent

The babesia organism was named after Viktor Babes, who first recognized it in blood cells of cattle in the late 19th century. Babesiosis was also the first vector-borne disease to be described in the literature by Smith and Kilbourne, as the etiologic agent of Texas cattle fever. *Babesia* shares a close affinity with malaria parasites in its intraerythrocytic niche in the host, which can and has led to its being mistaken for *Plasmodium* spp., the malaria agent (20, 45). It differs significantly from the genus *Plasmodium* in a number of features. First, there is no exoerythrocytic stage in its development as there is in the case of *Plasmodium*, although this is now open to question. Mehlhorn and Schein (38) have suggested that *B. microti* and *B. equi* initially invade lymphocytes prior to invading erythrocytes, similar to the development of *Theileria* spp. Subsequently, Mehlhorn and Schein have shown that *B. equi* indeed initially invades lymphocytes, serving as the basis for their reclassification of *B. equi* as *T. equi* (39). Additionally, *Babesia* divides by binary fission and not schizogony, develops within the cytoplasm of the host erythrocyte and not in a parasitophorous vacuole as does the malaria parasite, and does not form the characteristic pigment (hemozoin) in infected
erythrocytes as does Plasmodium. The insect vectors for Plasmodium spp. are anopheline mosquitoes, while Babesia spp. are transmitted by ticks, either transovarially for the large babesias or transstadially (from egg to larva) for B. microti (6, 38, 39, 50).

Diagnosis of Infection

A variety of factors are taken into account in diagnosing babesiosis, most importantly patient history and examination of blood smears. Table 1 presents these factors, and some additional ones, giving the relative pros and cons for each as a diagnostic tool. Detection of Babesia infections is most often by examination of stained blood smears (Fig. 1). There are, however, drawbacks in reliance upon blood smears alone. Identification of the parasites in thin blood films requires a reasonable level of parasitemia, otherwise the parasites might be missed. Some Babesia spp., because of their small size, might not be seen in thick blood smears. Inoculation of hamsters with samples of blood (xenodiagnosis) suspected of being infected with B. microti, for example, is also useful, especially when the level of parasitemia is low. This approach, however, is time-consuming, requiring 7 to 10 days before parasites are visible (15). Gerbils and splenectomized calves have been used for diagnosis of B. divergens infections (15). SCID mice, whose erythrocytes had been replaced by human erythrocytes, were

<table>
<thead>
<tr>
<th>Technique</th>
<th>Diagnostic method</th>
<th>Relative merit(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient history</td>
<td>Clinical signs and symptoms</td>
<td>Possible overlap with other types of infections (e.g., borreliosis)</td>
</tr>
<tr>
<td></td>
<td>Presence in region of endemcity</td>
<td>Regional occurrence of infections</td>
</tr>
<tr>
<td></td>
<td>Tick bites; contact with reservoirs</td>
<td>Individuals may not recall tick bites</td>
</tr>
<tr>
<td></td>
<td>Splenectomy</td>
<td>Asplenic individuals are at higher risk for infection</td>
</tr>
<tr>
<td></td>
<td>Blood transfusion</td>
<td>Low risk of infection</td>
</tr>
<tr>
<td></td>
<td>Advanced age</td>
<td>Elderly individuals are at higher risk for infection</td>
</tr>
<tr>
<td>Microscopic examination</td>
<td>Blood smear</td>
<td>Rapid, inexpensive, reliable if parasitemia is high; possible misdiagnosis as malaria</td>
</tr>
<tr>
<td></td>
<td>Immunofluorescent antibody technique</td>
<td>Generally requires reference laboratory; possible cross-reactivity with other protozoan pathogens producing false-positive reactions</td>
</tr>
<tr>
<td>Xenodiagnosis</td>
<td>Hamster inoculation</td>
<td>Effective but slow, requiring weeks for results; more-fastidious isolates might not be detected</td>
</tr>
<tr>
<td>In vitro cultivation</td>
<td>MASP culture</td>
<td>Amplification of infections with low parasitemia; impractical for human diagnostic purposes</td>
</tr>
<tr>
<td>Molecular techniques</td>
<td>PCR assay</td>
<td>Reliable and accurate if done properly; requires reference laboratory</td>
</tr>
</tbody>
</table>

FIG. 1. Giemsa-stained blood smear of a WA-1 type Babesia in human red blood cells showing intracellular morphological variety: Abbreviations: A, ring form; B, ameboid shape; C, tetrad configuration. The bar in the illustration represents 10 μm. Copyright Anne Kjemtrup.
used to diagnose babesiosis caused by a *B. microti*-like parasite in an asymptomatic human (55). Indirect immunofluorescence is also used in diagnosis. However, since cutoff titers vary between species of Babesia and the infected host, it is necessary that cutoff values be carefully established by each testing laboratory (33). Cross-reactivity of surface antigens may occur between *Babesia* spp., as well as between *Babesia* and *Plasmodium* (15), making it potentially difficult to identify different species or to clearly distinguish babesiosis from malaria.

In vitro cultivation of *Babesia* has also been used as a means of amplifying infections in animals in which parasites might have otherwise been missed. Thomford et al. (51) were able to cultivate babesias from blood of wild bighorn sheep and mule deer whose parasitemias were <0.01% and not detectable in stained blood smears (see below). Likewise, Holman et al. established *Babesia* cultures from a white-tailed deer (25) and from four horses (24) with low parasitemias (1 infected erythrocyte/6,000 in the deer) or negative blood smears (horses). In a later report, Holman et al. (21) established *B. equi* in continuous culture from naturally infected working horses from an area of endemicity. No parasites were detected in blood smears of the animals, nor did the animals exhibit clinical signs of babesiosis, but cultures were established from 15 out of 23 samples. Cultures were initiated from small amounts (0.2 ml) of packed erythrocytes processed in a microcentrifuge tube (26). PCR has been used effectively to diagnose infections as well as to monitor existing infections (26, 43).

Cryopreservation has been used for long-term storage of cultures (6, 26). Polyvinylpyrrolidone-40 is the cryoprotectant of choice; dimethyl sulfoxide or glycerol when used can cause osmotic shock to babesias at the time of thawing (42). Holman et al. (25) reported parasitemias of 17% after introduction of frozen *B. odocolei* into cultures, although *Babesia* spp. vary in their abilities to establish in vitro after freezing (6). A strain of *B. gibsoni* (canine parasite) was kept following cryopreservation in liquid nitrogen for 19 years (61). A frozen stablile of *B. microti* (ATCC 30221) is available from the American Type Culture Collection (http://www.atcc.org) which can be maintained in vivo in the golden (Syrian) hamster.

**Babesia in Erythrocytes**

In general, there are small (diameter, 1.5 to 3 µm) and large (diameter, 3 to 5 µm) *Babesia* species, although the size of the parasite may reflect the size of the host’s erythrocytes (20, 38). Additionally, the size of the parasite can determine the number of division products (merozoites) developing within an erythrocyte—two for large babesias and four for the small species (50). The same parasite, *B. divergens*, from humans, can assume different positions in erythrocytes of different hosts, from subcentral in human blood cells to central in those of the ox (14). Thus, it would appear that the host erythrocyte dictates the location of the parasite.

The presence of pairs or tetrads ("Maltese cross" pattern) of parasites in stained erythrocytes is diagnostic of babesiosis, although the tetrad configuration typical of *B. microti* is not commonly seen in blood smears (26, 50). The Maltese cross pattern is characteristic of the small babesias (e.g., *B. microti* and the recently described WA-1 type parasite from the western United States) and results in the formation of several infectious stages within the erythrocyte developing at the same time (26).

**Babesia and the Blood Supply**

Passive infection of humans with *Babesia* through blood transfusion is a potentially worrisome problem. Gerber et al. (11), in a study of patients having undergone cardiothoracic surgery with multiple blood transfusions, noted that the risk of acquiring babesiosis from packed erythrocytes was 0.17%, making it a potential but minor threat to the blood supply. Nevertheless, transmissions are now well-documented in the literature (7, 11, 17, 28, 47, 55).

**High-Risk Groups**

The first human *Babesia* infection was recognized in an asplenic farmer in Yugoslavia in 1957 (reviewed in reference 46). In Europe, human infections are caused by *B. divergens*, a parasite of cattle. In North America, human infections are more likely to be attributed to the rodent parasite, *Babesia microti*. Infections with *B. divergens* are likely to be more severe than those caused by *B. microti*, with a 42% mortality associated with *B. divergens* compared to a 5% mortality associated with *B. microti* in the United States (15). The majority of human cases of babesiosis occur in individuals >50 years old (43); asplenic individuals also represent a high-risk group. In the United States, most cases have occurred in the Northeast and Midwest, with ticks transmitting the parasites to humans from animal reservoirs. Meldrum et al. (40) reported on 136 cases in New York State between 1982 and 1991, with the highest incidence among elderly men (>80 years) and women (>70 years); 5% of the New York cases were fatal. Cases have appeared on the West Coast (Washington State and California), but the tick and animal reservoirs have yet to be defined.

**Phylogenetic Relationships**

Identification of species of babesias was predicated upon a high degree of host specificity (46). In recent years, however, recognition of human cases of bovine, equine, and rodent origins has suggested that host restriction is not as confining as it was once assumed (20). Indeed, the isolation of babesias that do not fit neatly into the recognized groups has further blurred species and even generic distinctions (17, 26, 43). Human infections occurring on the West Coast of the United States have been caused by *Babesia*-like organisms designated WA-1 type *Babesia* (where the prefix "WA" stands for Washington State, where the first human case was described [45]). Based upon sequencing data, WA-1 type *Babesia* shows more affinity to small babesial isolates from dogs and wildlife in California than to *B. microti* (30, 31). A recent human isolate from Missouri, MO-1, showed affinity to the cattle parasite *B. divergens* (18), and a cluster of Wisconsin cases were caused by *B. microti*, the rodent parasite (19). In addition, a *B. microti*-like species (PB-1) has been isolated from baboons, infecting as many as 40% of animals in the colonies (4, 26).

The sequencing of small subunit ribosomal DNA (ssrDNA) has altered the classic taxonomic schemes, which were based largely on host specificity and morphology, for *Babesia*, as well
as *Theileria*. *B. divergens* and *B. gibsoni*, members of the “small” group, fit better with the “large” representatives of the genus based on their ssrDNA sequence (26, 30, 31). The small babesias (*B. microti*, *B. felis*, and *B. rodhaini*) fit among members of a group separate from the large babesias and are sister to the genus *Theileria* in the ssrDNA phylogenetic trees (30). Kjemtrup et al. (30, 31), basing their results on sequencing data, concluded that human and wildlife isolates cannot be readily distinguished from one another. The implication of this finding is that a variety of mammals might serve as reservoirs for organisms that cause human infections. It is likely that as additional human babesias are isolated and sequenced, further changes will occur in the taxonomy of the genus.

Generalized treatments of *Babesia* are found in reviews by Homer et al. (25), Kjemtrup and Conrad (29), Mahoney (37), Mehlhorn and Schein (38), Persing and Conrad (43), Ristic (26), and Lewis (46), and Telford et al. (50). Earlier reviews of cultivation of the parasites are found in the works of Canning and Lewis (46), and Telford et al. (50, 51), basing their results on sequencing data, concluded that human and wildlife isolates cannot be readily distinguished from one another. The implication of this finding is that a variety of mammals might serve as reservoirs for organisms that cause human infections. It is likely that as additional human babesias are isolated and sequenced, further changes will occur in the taxonomy of the genus.

**CULTIVATION OF BABESIA**

Since techniques for culturing *Babesia* have been developed for species that infect animals, these are reviewed in this section. Bear in mind that some of these same species are capable of infecting humans, so that the same culture methods would be applicable to human or animal parasites.

### Basic Culturing Techniques

Some general features of in vitro cultivation include defibrination and washing of blood cells used in culture and addition of antibiotics to the culture medium. With regard to the latter, Holman et al. (25) noted that they have used a variety of antibiotics in their media, including penicillin, streptomycin, garamycin, and amphotericin B (Fungizone), without affecting parasite growth. Given the similar niches of the malaria parasite and *Babesia*, one might suppose that the method developed by Trager and Jensen (53) for cultivation of *Plasmodium* would work for *Babesia*; this has not been the case (8, 35). Sera used as medium supplements for in vitro growth of Babesia spp. represent a variable factor, making it necessary to screen batches for lots that support parasite growth (48, 58). Fetal calf serum may actually inhibit growth of *Babesia* spp. (6, 36), although this has not been invariably found to be the case (3).

Table 2 summarizes the different types of basal media and buffers used for cultivation of *Babesia* spp. reviewed below and indicates the type of serum (or serum-free culture) used.

<table>
<thead>
<tr>
<th>Babesia species or strain</th>
<th>Medium</th>
<th>Serum type (concn [%])</th>
<th>Parasitemia</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. bovis</em></td>
<td>HEPES-buffered medium 199</td>
<td>Bovine (40)</td>
<td>38%</td>
<td>35, 36</td>
</tr>
<tr>
<td><em>B. caballi</em></td>
<td>HEPES-buffered HL-1</td>
<td>Serum-free, plus lipid-rich supplements</td>
<td>4-6%</td>
<td>62</td>
</tr>
<tr>
<td><em>B. divergens</em></td>
<td>HEPES-buffered RPMI 1640</td>
<td>Human (10)</td>
<td>&gt;60%</td>
<td>14, 16</td>
</tr>
<tr>
<td><em>B. equi</em></td>
<td>HL-1</td>
<td>Fetal calf (20) plus serum factors</td>
<td>&gt;15%</td>
<td>22</td>
</tr>
<tr>
<td><em>B. gibsoni</em></td>
<td>HL-1</td>
<td>Dog (20)</td>
<td>1-6%</td>
<td>61</td>
</tr>
<tr>
<td><em>B. microti</em></td>
<td>RPMI 1640</td>
<td>Fetal calf (30–40)</td>
<td>2-fold increase</td>
<td>48</td>
</tr>
<tr>
<td><em>B. odocoilei</em></td>
<td>TES-buffered medium 199</td>
<td>Deer (20) or bovine (40)</td>
<td>31%</td>
<td>25</td>
</tr>
<tr>
<td><em>B. rodoceri</em></td>
<td>TES-buffered medium 199</td>
<td>Fetal calf (40)</td>
<td>20-30%</td>
<td>52</td>
</tr>
<tr>
<td>Strain WA-1</td>
<td>TES-buffered medium 199</td>
<td></td>
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</tr>
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</table>

In one of the first attempts to establish continuous cultures of babesias, Erp et al. (8, 9) used an agitated culture technique for the cattle parasite *B. bovis*. The medium consisted of HEPES-buffered tissue culture medium 199 (50%) supplemented with freshly collected bovine serum (50%), at pH 7. Erythrocytes were added to give a cell volume of 20 to 30%, and the entire suspension was stirred in a spinner flask at 100 rpm. The cultures were kept at 37 to 38°C in a 5% CO2–95% air atmosphere and required daily subculturing. Using this system, babesias increased to an estimated 5 × 107 in the course of continuous cultivation (9). The percentage of infected erythrocytes after six subcultures was 10 to 15%, compared to <3% after 24 to 48 h using the Trager and Jensen technique. Parasites retained their virulence for causing disease in cattle. In a subsequent paper, the same authors (10) explored culture variables and found no difference between the media RPMI 1640, 199, and NCTC-135 in supporting growth but found that pH was a critical factor in growth. A shift in pH up (to 7.3) or down (to 6.7) adversely affected parasite growth. They noted that the parasite would grow in Hanks’ salts with serum at 40% but that growth was better with the added components found in tissue culture media.

Continuous growth of *B. bovis* with a similar culture medium was achieved by Levy and Ristic (35) using a stationary layer of erythrocytes rather than a suspension culture. Called the microaerophilous stationary phase (MASP) culture technique, the parasites proliferate in a settled layer of blood cells. The MASP system is regarded as the more convenient for parasite growth (6, 13). The culture medium was HEPES-buffered medium 199 (60%) and bovine serum (40%), and an inoculum of infected and normal erythrocytes was used, adjusted to pH 7. The suspension was placed in culture vessels in a ratio of 0.62 ml of suspension/cm² of culture surface, or a depth of 6.2 mm. (A table in reference 6 calculates appropriate volumes of blood cells, medium, and serum to obtain the desired volume in growth medium.) Cultures were incubated at 37 to 38°C in an atmosphere of 5% CO2–95% air. The depth of the fluid in the culture vessel was critical to successful parasite replication. Cultures supporting growth of parasites turned dark red to black due to oxygen utilization by the parasites and deoxygenation of hemoglobin, with the pH of the culture rising to 7.2 from its initial pH of 7. Cultures with a bright red color did not support parasite reproduction. Medium was replaced at daily intervals, and subculturing was performed at 48 to 72 h. They calculated that the cumulative increase in parasites over the 83-day period was about 1.7 × 10²³, with a maximum para-
sitemia of about 38% and an approximate doubling time of 7 to 10 h (36).

Goff and Yunker (12) used a modification of the MASP technique to culture B. bovis in medium 199 (60%) and normal adult bovine serum (40%). In determining the percentage of parasitized erythrocytes (PPE), they distinguished dense forms (parasites without visible cytoplasm), trophozoites (conventional parasites), and merozoites (paired pyriform bodies) in stained blood smears. They found that the duration of the exponential growth phase varied inversely with the initial PPE. Replication of parasites ceased when the culture reached a threshold level, and medium changes alone would not allow continued growth. Based on the growth parameters used, they estimated a maximum of 3 × 10⁹ to 4 × 10⁹ infected blood cells, with a PPE of 7%. Reducing the number of erythrocytes elevated the PPE to about 34%, but the absolute number of infected blood cells remained constant. They also pulse-labeled cultures for 12 h with [³H]hypoxanthine, using label uptake as a measure of parasite growth. Uptake of label was directly proportional to the PPE, with trophozoites responsible for the greatest uptake. In a later paper dealing with measures to make the cultivation process less labor-intensive, Goff and Yunker (13) examined variables affecting growth of B. bovis in continuous culture. They found that parasite growth was better at slightly alkaline pHs (pH 7.02 to 7.79) but that the organisms were intolerant of acidic pH conditions (pH 6.86), with optimal growth occurring within the range of 7.31 to 7.39. They reported that the buffer 3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid (TAPSO) (10 mM) was superior to N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), allowing growth through 4 days, while TES induced degenerative changes of the parasites after 3 days. They also compared medium changes at 24-, 48-, and 72-h intervals and found that daily medium changes were necessary to maintain logarithmic growth of parasites, suggesting that metabolic wastes accumulating in the medium dampen continued growth. Although cold storage of media has been reported to produce less-than-optimal parasite growth, refrigeration and/or freezing of medium had essentially no adverse affect upon growth (13). Figure 2 presents a flowchart for the isolation of B. bovis from cattle blood and establishment of a MASP culture.

**B. divergens**

A parasite primarily of cattle, B. divergens is largely responsible for human babesiosis in Europe. Väyrynen and Tuomi (54) established B. divergens in continuous culture from a strain previously maintained in splenectomized calves. They used medium 199 with bovine serum (40%), obtaining parasitemias of 5 to 10%.

Gorenflo et al. (14) established isolates from two humans with babesiosis from France, as well as three other human patients from European sources, using a modified Trager and Jensen technique with HEPES-buffered RPMI 1640 and human serum (10%) with a packed cell volume of 5%. Parasitemias of >60% were obtained, which they attribute to their use of lower concentrations of serum and hematocrit than those previously used by others cultivating B. divergens. The parasite was also shown to be able to infect gerbil, ox, and human erythrocytes equally well and to retain its virulence, indicating a low degree of host cell specificity.

Grande et al. (16) used one of the French isolates to establish the parasite in a serum-free culture. Beginning with RPMI 1640 with 10% human serum, they gradually adapted the strain to 0% serum concentrations, with a concomitant drop in PPE from between 40 and 50% to 3%. Addition of a vitamin mixture did not increase the low PPE nor did the addition of reduced glutathione, but replacement of serum restored PPE to normal levels. They tested various supplements as replacements for the serum component in the medium. Addition of Albumax I or bovine serum albumin (BSA) (Cohn’s fraction V), at concentrations of 5 and 10 g/liter, respectively, restored the PPE to >30%. Protein-extracted Albumax I and fatty acid-free BSA did not support parasite growth and led to erythrocyte lysis. Addition of lipid fraction from serum to lipid-free albumin produced a PPE of 7%, suggesting that specific lipids with a carrier (BSA) were needed for parasite growth and

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**FIG. 2.** Chart presenting a generalized scheme for the isolation of B. bovis into MASP culture from a bovine blood sample. The scheme presented was designed by using information presented in the works of Levy and Ristic (36) and Canning and Winger (6).
replication. Grande et al. (16) postulated that the parasites’ need for phospholipid associated with de novo membrane formation is the factor that limited growth in host erythrocytes and that this need can be satisfied only by an exogenous source of phospholipid and a carrier such as albumin. They note, however, that PPE was not restored to its normal level of 40 to 50%, indicating that serum provided other still-undetermined components needed for optimal growth of the parasite.

*B. divergens* adapted to growth in rat erythrocytes were maintained in continuous culture using the Trager and Jensen (53) technique by Ben Musa and Phillips (3), ultimately attaining in vitro parasitemias of up to 30%. The medium used was HEPES-buffered RPMI 1640 with 20% fetal calf serum.

Konrad et al. (32) utilized low temperatures to slow the schedule of subculturing for *B. divergens* and *B. major*. After allowing cultures to develop, the medium was changed and the cultures were shifted from 38 to 4°C. At this lower temperature, it was possible to delay subculturing for up to 7 days. Cultures could also be kept at room temperature for as long as 5 days without subculturing. Unlike the typical MASP culture in which the erythrocyte layer darkens, these cultures remained bright red, indicating oxygenation of hemoglobin. Cultures could be resurrected by shifting to 38°C upon subculturing.

*B. caballi*

*B. caballi* is an equid parasite that has been established in culture using RPMI 1640 and horse serum (reviewed in reference 62). As the starting point for achieving serum-free cultivation of two strains of *B. caballi*, Zweygarth et al. (62) used HEPES-buffered HL-1 medium (80%) with glutamine and hypoxanthine and horse serum (20%), with horse erythrocytes as host cells. As potential serum replacements, they tested BSA (5, 10, and 20 mg/ml), chemically defined lipids (0.25, 0.5, 0.75, and 1%), Albumax I (5, 10, and 20 mg/ml), and combinations of these substances in media. Parasites were adapted to the serum-free state by progressive reduction of serum upon subculturing. Lipid-rich Albumax I replaced serum at all concentrations, but not BSA or the chemically defined lipid preparation. Combinations of Albumax I (10 mg/ml) and chemically defined lipids (0.25 and 0.5%) gave optimal PPE of ca. 4 to 6%, but not a combination of BSA and lipids. Replacement of HL-1 medium by either RPMI 1640 or by Dulbecco’s modified Eagle medium–F-12 did not support parasite growth.

*B. equi*

*B. equi* was cultured in vitro by Holman et al. (22). Unlike most other *Babesia* spp., this species exhibits exoerythrocytic schizogony. They employed HL-1 as the basal medium, with Albumax I (1 mg/ml), 2% HB101, glutamine, and 20% fetal calf serum as supplements. After establishment of cultures, fetal calf serum could be replaced by normal adult horse serum. Initially cultured in 5% CO₂-95% air, the cultures were shifted to 2% O₂-5% CO₂-93% N₂ when the cells appeared to be in poor condition. Medium HL-1 contains a variety of factors, including insulin, transferrin, and saturated and unsaturated fatty acids, not found in most tissue culture media (22).

Zweygarth et al. (59) used TAPSO-buffered medium 199 with 40% horse serum and supplemented with glutamine and hypoxanthine to culture a strain of *B. equi* in a 5% CO₂-95% air atmosphere. Parasitemias of 10 to 25% were produced. Parasite growth was not supported by lower serum concentrations (10 and 20%) or heat-inactivation of serum (56°C for 30 min). Hypoxanthine could not be omitted from the medium, but could be replaced by adenosine and guanosine.

Subsequently, Zweygarth et al. (60) established the same strain of *B. equi* in serum-free culture using HEPES-buffered HL-1 medium supplemented with glutamine and hypoxanthine. Parasite cultures were adapted to serum-free conditions by progressive subculturing in the absence of serum, with a baseline PPE of 2 to 4%. Parasite growth did not occur in the basic medium by itself. Optimal growth (PPE of 13%) of the parasite occurred with 1% chemically defined lipids and BSA at 5 mg/ml, but growth also occurred with BSA alone (at 5 mg/ml, PPE was 9%).

*Babesia* from Wild Artiodactyls

Holman et al. (25) isolated *B. odocoilei* from a white-tailed deer (*Odocoileus virginianus*) using the MASP technique. The isolate was established in TES-buffered medium 199 with glutamine and either deer (20%) or bovine (40%) serum. At initiation of culture, parasitemia was too low to quantitate accurately, but it rose to ca. 2% by day 7, to ca. 12% by day 11, and ultimately achieved 31% by the fifth subculture. Deer serum used in the primary culture appeared to support better growth (about 11%) than did bovine serum (ca. 4%).

Babesias from desert bighorn sheep and mule deer were isolated by Thomford et al. (51) in medium 199 with fetal calf serum (40%). Erythrocytes used as host cells were obtained from bighorn sheep, mule deer, and domestic sheep. Established cultures were maintained in a 5% CO₂-95% air atmosphere. Isolates from bighorn sheep included both large and small babesias, while the mule deer isolate was of the large variety. The size of the babesias remained consistent throughout cultivation. Variations among the isolates were found regarding the suitability of different host erythrocytes. Growth of the isolates was generally better in mule deer erythrocytes than in bighorn or domestic sheep cells. Two of the small babesias gave parasitemias of ca. 20% with mule deer erythrocytes, about twice the level of parasitemia found in sheep cells. Parasitemias of the large isolates were lower (<14%). *B. odocoilei* (see above) from cryopreserved samples did not grow in the three different host cells used in this study but did grow in erythrocytes from the white-tailed deer.

In a first report of isolation of *Babesia* from an elk (*Cervus elaphus*), Holman et al. (23) cultivated the parasite in HL-1 medium with 20% bovine serum, supplemented with glutamine, and white-tailed deer erythrocytes. Parasitemias of 3 to 19% were obtained. The isolate showed immunofluorescence reactivity with *B. odocoilei* from the white-tailed deer and *B. divergens*, but not to other *Babesia* isolates (from bighorn sheep and caribou), including *B. bovis*.

Other *Babesia* spp.

Several other species of *Babesia* have been cultivated, although not to the extent of those previously described. *B. microti*, a species that infects humans, has been cultured by
Bautista and Kreier (1) using the candle jar technique of Trager and Jensen for cultivation of Plasmodium (53). Short-term cultures were established from infected hamsters using fetal calf serum and hamster erythrocytes. The system was used to evaluate the effects of immune versus normal sera on parasite growth. Parasitemia in normal hamster serum was about 9%, compared to about 3% in immune serum. Hamster or rat sera supported parasite growth as well as fetal calf serum, but rat erythrocytes did not support parasite growth. The authors concluded that it is the erythrocyte and not the serum that is a determinant in ability of babesias to infect a host. Short-term cultures of B. microti were also used to study behavior of macrophages in the presence of immune vs. normal hamster sera (2).

B. microti, as well as B. rodhaini (rodent parasite), was grown in RPMI 1640 with 30 to 40% fetal calf serum, in a 3% CO₂–8% O₂ gas mixture (48), with variation due to the source of calf serum used in the medium. Cultivation of B. bigemina (parasite of cattle) is reviewed by Canning and Winger (6).

B. gibsoni, an agent of babesiosis in canines, was grown using the MASP technique in HL-1 medium supplemented with t-glutamine, dog serum, and dog erythrocytes in an atmosphere of 5% CO₂–2% O₂–93% N₂ (61). Yamasaki et al. (57) used modified Eagle medium and both high- and low-potassium dog erythrocytes for cultivating B. gibsoni. They reported that high-potassium erythrocytes, as well as lysates of these cells, supported better growth of the parasite than did low-potassium cells. Growth was enhanced by aspartate, glutamate, and glutathione.

Babesia-Like Isolates

Babesia-like isolates are the blood parasites which, like the WA-1 isolate from Washington State (17, 45, 52) and the isolates from California (44), are distinct from known Babesia spp. that are normally found in humans. Sequencing data indicate that these isolates and other Babesia spp. (B. equi of horses and B. gibsoni of canids) have a close phylogenetic relationship with members of the genus Theileria (43). An implication of this information is that organisms like WA-1 may, like Theileria, have an intralymphocytic stage in their development (52). The presence of a lymphocytic stage in the development of B. equi has argued for its inclusion among Theileria spp. (5, 6, 39).

Thomford et al. (52) used TES-buffered medium 199 at pH 7.0 (60%) and fetal calf serum (40%), with added glutamine, to culture the WA-1 human isolate from Washington State. They initially used an atmosphere of 2% O₂–5% CO₂–93% N₂. Cultures, once established, were transferred from 24-well plates to 25-cm² tissue culture flasks in a 5% CO₂–95% air atmosphere. The cultured isolate retained its morphological appearance and its virulence after 6 months in vitro. Partial medium replacement was done on a daily basis, and subculturing was performed at 2- to 3-day intervals when the PPE reached 20 to 30%.

Theileria spp.

The Theileria sp. piroplasms are noted in passing here because of the overlap between some apparent Babesia and Babe-

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CULTURE-RELATED FACTORS

Culture-Derived Soluble Antigens

Working with B. bovis, James et al. (27) utilized the MASP culture technique to demonstrate antigens (exoantigens) released by the parasites in culture. Three distinct proteinaceous antigens (molecular weight, 37,000 to 40,000) were recognized by immunoelectrophoresis of culture supernatants. One of these was reported to be derived from the surface coat of the merozoite, shed as parasites penetrated into the erythrocyte. These culture-derived antigens have been demonstrated under conditions under which they would not be subjected to either immunological or physiological responses of the host. In another study making use of cultured B. bovis, it was shown that supernatants derived from Babesia antigen-treated monocyte cultures were inhibitory for B. bovis cultures (41). Both culture-attenuated live babesias and exoantigens were used to immunize cattle, with the former being more protective than the latter (49).

Strain Attenuation

For many pathogenic organisms, virulence diminishes in cultured isolates. The same appears true for Babesia isolates (6). The loss of virulence with culture passage is of particular use in developing strains of babesias that might serve for immunization of susceptible hosts (34, 49). Yunker et al. (58) cultured a virulent isolate of B. bovis using the MASP technique, gradually replacing the bovine serum additive with horse serum, until one line was grown in 40% horse serum. This line was tested in splenectomized calves against a virulent isolate. The culture-derived line produced a relatively mild reaction in the host, which protected calves against challenge by the virulent B. bovis strain. In a subsequent study, Kuttler et al. (34) confirmed reduction of virulence of the in vitro-grown strain when inoculated into cattle, with only one of twelve animals showing parasitemia. None of the 12 animals showed signs of disease when challenged with a virulent B. bovis strain. Winger et al. (56) attenuated a strain of B. divergens following 18 months in culture, successfully using it to protect gerbils from challenge with a virulent strain of the parasite.

Not all cultured babesias, however, become attenuated. Gorencloot et al. (14) reported no loss in virulence of a strain of B. divergens after 2 years in culture. B. divergens, adapted to growth in rat erythrocytes, retained virulence for splenectomized rats after continuous in vitro growth (3).
Screening of Antimicrobial Agents

A decidedly useful adjunct of in vitro cultivation of Babesia, which has been only moderately exploited, is testing the efficacy of antimicrobial compounds against the parasite.

CONCLUSIONS

There is a steadily increasing body of literature dealing with Babesia based on two factors: (i) that the organism is now recognized as a zoonotic parasite, with humans acquiring infections from mammalian animal reservoirs, and (ii) that the recognized as a zoonotic parasite, with humans acquiring in infections from mammalian animal reservoirs, and (ii) that the organism is now based on two factors: (i) that the organism is now moderately exploited, is testing the efficacy of antimicrobial compounds against the parasite.

REFERENCES


