MOLECULAR AND IN VITRO CHARACTERIZATION OF A *Babesia divergens*-LIKE AGENT FROM EASTERN COTTONTAIL RABBITS (*Sylvilagus floridanus*) ON NANTUCKET ISLAND

A Thesis

by

ANGELA M. SPENCER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2005

Major Subject: Veterinary Parasitology

MOLECULAR AND IN VITRO CHARACTERIZATION OF A *Babesia divergens*-LIKE AGENT FROM EASTERN COTTONTAIL RABBITS (*Sylvilagus floridanus*) ON NANTUCKET ISLAND

A Thesis

by

ANGELA M. SPENCER

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Approved by:

Chair of Committee,	Patricia J. Holman
Committee Members,	Karen F. Snowden
	Thomas M. Craig
	Clint Magill
Head of Department,	Ann B. Kier

August 2005

Major Subject: Veterinary Parasitology

ABSTRACT

Molecular and In Vitro Characterization of a *Babesia divergens*-like Agent from Eastern Cottontail Rabbits (*Sylvilagus floridanus*) on Nantucket Island. (August 2005)

Angela M. Spencer, B.A., Baylor University

Chair of Advisory Committee: Dr. Patricia J. Holman

A *Babesia* sp. isolated from eastern cottontail rabbits (*Sylvilagus floridanus*) is morphologically similar and genetically identical, based on SSU rRNA gene comparisons, to two agents responsible for human babesiosis in North America and is closely related to the European parasite, *Babesia divergens*. The ribosomal RNA (rRNA) internal transcribed spacer regions (ITS1 and ITS2) and the 5.8S rRNA genes of *Babesia* isolates were sequenced and analyzed. The rRNA ITS region sequences of three isolates, one each from Kentucky, Massachusetts and Great Britain, considered *Babesia divergens*-like organisms, were compared to two *Babesia microti* isolates, two *Babesia odocoilei* isolates and a well defined *Babesia divergens* isolate. The two *B. divergens*-like isolates from North America shared identical rRNA ITS1-5.8S-ITS2 region sequences, and the clones of these isolates clustered into one clade in three phylogenetic analyses, suggesting that these isolates are conspecific. In vitro comparison of host erythrocyte specificity between the rabbit *Babesia* sp. and *B. divergens* was employed to discriminate between the two organisms and to determine the usefulness of in vitro techniques for *Babesia* sp. characterization. In vitro growth of the rabbit *Babesia* sp. was supported in human and cottontail rabbit erythrocytes, but not in bovine cells.

Babesia divergens in vitro growth was supported in human and bovine erythrocytes, but not in cottontail rabbit cells. Morphological characteristics and size differences also distinguished the two parasites from one another. The erythrocyte specificity and parasite size differences reported in this study agree with previous in vivo results and validate the use of in vitro methods for characterization of *Babesia* species.

ACKNOWLEDGEMENTS

I have grown in many ways during the past three years (mentally and emotionally, not vertically) and I am grateful to many people for my progress. I would like to thank my committee chair, Dr. Holman, who provided me with an interesting project and taught me more than I ever thought I would know about in vitro cultivation of *Babesia*. The remaining members of my committee, Drs. Snowden, Criag, and Magill, have been wonderfully helpful, and I have appreciated the interest they have shown in my work.

I also would like to acknowledge my parents, brother Bill, Uncle Stephen and grandparents who have always been supportive of me and my pursuits. I could not have made it here without them. Maybe now that I am not babysitting parasites every day, I will be able to see them once and a while.

Finally, I would like to thank the dear friends that I have made here at TAMU, without whom I probably would have given up. In addition to providing nourishment and Monday-night entertainment, Paulette and Jan have been wonderful, supportive friends that have held me up when I needed it. I am also grateful for the friendship I share with Allison. I appreciate the logic she instills into my manic moments and her compensating for whichever side of my brain happens to be malfunctioning. I must be sure to thank Kylie for the time she took to teach me lab techniques and always remembering me while she has been away. Last but not least, Thomas, who brightens my days.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Members of the Apicomplexan genus *Babesia* are notable as a cause of human and veterinary disease as well as for the impact they have had on the current understanding of parasitology. These protozoan parasites infect vertebrate erythrocytes causing severe to chronic anemia in susceptible hosts. Biblical descriptions suggest that Egyptian cattle exhibited signs similar to those currently recognized as cattle babesiosis or redwater fever caused by *Babesia bovis* infection (Homer et al. 2000). *Babesia bovis* and *Babesia bigemina* are both responsible for the devastating babesiosis outbreak that occurred in Texas cattle during the mid-1800s and resulted in the pivotal discovery by Smith and Kilbourne (1893) that *B. bigemina* is transmitted by the tick vector *Boophilus annulatus*. This discovery was the first proof that arthropods could vector a disease agent and has contributed significantly to the present understanding of vector borne disease (Smith and Kilbourne 1893; Levine 1985).

In the past century, additional species of *Babesia*, susceptible hosts, and competent vectors have been identified. A wide variety of mammals are susceptible to the disease, including equids, ungulates, and humans worldwide. Increased physician awareness has caused an increase in the number of diagnosed human babesiosis cases. Ecological changes and increased outdoor activities have contributed to increased prevalence by bringing humans into contact with tick vectors more often. The rise in babesiosis diagnoses has re-defined the infection as an emerging zoonotic disease (Gorenflot et al.1998; Telford and Goethert 2004; Kjemtrup and Conrad 2000).

This thesis follows the style of Parasitology Research.

Babesia organisms can be visualized in a Giemsa stained infected blood smear at 1000X magnification. They are pyriform (pear shaped) or seen as round or amoeboid forms (Levine 1985). Depending on *Babesia* species, a host erythrocyte may be parasitized by single, paired, or multiple organisms. The size of the organisms varies depending on the species and are therefore classified as either small (1.0-2.5 µm) or large (2.5-5.0 µm) *Babesia*, accordingly (Levine 1985). Electron microscopy reveals the presence of an apical complex, micronemes, rhoptries and other organelles characteristic of the phylum Apicomplexa (Simpson et al. 1963; Simpson et al. 1967; Droleskey et al. 1993; Holman et al. 2005)

The *Babesia* species sensu stricto, or true *Babesia*, are characterized by transovarial transmission by the vector tick and the limitation of infecting only erythrocytes in the mammalian host (Levine 1985). Species within this classification include *Babesia divergens*, *B. bovis* and *Babesia odocoilei* (Levine 1985). Transovarial transmission occurs when developing parasites invade the ovaries of the tick vector and infect the oocytes within. These infected eggs hatch, the larval ticks mature and are then capable of transmitting the parasites during feeding without having previously ingested an infected blood meal. Transovarial transmission is an efficient method of parasite survival in that thousands of developing ticks can become infected while in the ovary of the female tick. Furthermore, the existence of ticks that have become infective early in life creates more opportunities for the tick to transmit the parasite to competent vertebrate hosts. The life cycle of *B. divergens* utilizes transovarial transmission and benefits from the resulting infectivity of all life stages of the tick vector, *Ixodes ricinus.* The larvae and nymphs of this tick species feed on small animals, predominantly rodents and ground

dwelling birds. Adult ticks are capable of climbing higher onto vegetation and therefore parasitize larger animals including deer, sheep, and cattle (Wall and Shearer 2001). Transstadial transmission may also occur through which an individual tick becomes infected during a blood meal and remains infective through the following molts. During this stage to stage transmission, the parasites multiply into clumps of up to 200 organisms, referred to as pseudocysts, within tick phagocytes. Approximately 11-16 days following a blood meal, these spherical pseudocysts become club shaped, exit the cells and migrate to tick muscle tissue. The organisms divide but remain in the tick muscle, which remains unchanged during the molting process, until induced by tick feeding, during the next stage, to migrate to the salivary glands (Levine 1973).

Babesia microti and *Babesia equi* are atypical within the genus *Babesia* due to the ability to undergo a pre-erythrocytic stage in host lymphocytes and the inability to be transmitted transovarially (Mehlhorn and Schein 1984). The transmission of *B. microti* through the tick vector *Ixodes scapularis* exemplifies the transstadial route of transmission. The larval ticks become infected during a blood meal on the white footed mouse (*Peromyscus leucopus*), the preferred host for this stage. Following a molt, the nymphs feed indiscriminately and are most responsible for transmitting the parasite to humans (Healy et al. 1976; Gorenflot et al. 1998). Adults of *I. scapularis* preferentially feed on white tailed deer (*Odocoileus virginianus*), which are incompetent reservoirs for *B. microti,* but are very important in completing the life cycle of the vector tick (Peisman et al. 1979).

Babesia undergo asexual replication in the vertebrate intermediate host and both asexual and sexual replication in the definitive host Ixodid ticks (Mehlhorn and Schein 1984; Kjemtrup and Conrad 2000). The parasites enter the tick during the blood meal and are carried to the tick gut, where they become sexually competent cells by gamogony. The resulting zygotes then penetrate into the epithelial cells of the gut before entering the hemolymph, which carries the organisms to the various tick cells including fat bodies and nephrocytes and eventually the salivary gland acini. Sporogony, a form of asexual replication, occurs within the salivary gland cells, and is followed by specialized organelle development and a budding process, which results in mature sporozoites. The vector tick undergoes a molt before becoming infective to a vertebrate host during which the parasite is maintained in the tick mesoderm (Levine 1973). Parasite transmission is possible once the tick takes a blood meal following metamorphosis. During the final hours of tick attachment, sporozoites are in the lumen of the salivary gland and available for injection into the vertebrate host (Homer et al. 2000; Levine 1985; Mehlhorn and Schein 1984).

Once in the vertebrate host, the sporozoites invade erythrocytes. Within erythrocytes the sporozoites differentiate into trophozoites and divide into merozoites, then exit the cell and each merozoite enters a new erythrocyte to begin another cycle of multiplication. Using transmission electron microscopy (TEM), Droleskey and others (1993) demonstrated that within the erythrocyte, *B. odocoilei* merozoites divide through either binary fission or a nuclear budding process resembling that described by Rudzinska and Trager (1976) for *B. microti*. Through nuclear budding, a single parasite is capable of producing up to eight progeny in two rounds of division (Droleskey et al. 1993;

Rudzinska and Trager 1976). The resulting daughter cells mature to merozoites, which continue to infect additional erythrocytes. Once ingested by the tick vector, trophozoites may mature into gametocytes, in response to the decrease in temperature, which are capable of sexual reproduction (Homer et al. 2000).

There are two named species in the genus *Babesia* that have traditionally been identified in human cases of babesiosis and are considered to be of zoonotic risk in endemic areas. *Babesia divergens* was identified as the cause of the first reported human case of babesiosis in 1957, which occurred in a splenectomized Yugoslovian farmer (Skrabolo and Deanovic 1957). Since then, approximately 30 European cases have been reported spanning from Eastern Europe to Italy and Great Britain (Piccaluga et al. 2004; Gorenflot et al. 1998). *Babesia divergens*, is vectored by the tick *I. ricinus* and is an economically important pathogen of cattle in Europe. Cattle infected with *B. divergens* experience reduced milk production and poor weight gain, but rarely death (Gorenflot et al. 1998). The human cases occurred most often in rural areas where cattle are present and corresponded to the seasonal activity of the tick vector (May-September). Eighty three percent of the documented European patients have been asplenic, having a high fatality rate. The broad range of symptoms, which include fever, fatigue, chills, hemoglobinuria, thrombocytopenia, hepatomegaly, and splenomegaly, are the result of the parasite-induced hemolytic anemia (Homer et al. 2000).

As described for the true babesias, *B. divergens* undergoes transovarial transmission through the vector tick and does not undergo a pre-erythrocytic stage in the vertebrate host (Levine 1985; Holman et al. 2000). Morphologically, *B. divergens* is a small

parasite (1.5 x 0.4 µm) most commonly seen in pairs, which diverge widely from each other near the circumference of the infected bovine erythrocyte (accolé form), but also can be seen as single round parasites and rarely as tetrads (Levine 1985). In human erythrocytes, the pairs are seen in central or subcentral portions of the cell (Gorenflot et al. 1998). Although Gorenflot et al. (1998) did not mention the accolé form, this form has been noted in the *B. divergens*-like cases infecting humans in North America (Herwaldt et al. 1996; Beattie et al. 2002).

The rodent *Babesia*, *B. microti*, is recognized as the etiologic agent of Nantucket fever of humans in the United States and is reported to infect humans on other continents as well (Levine 1985; Herwaldt et al. 2004; Kjemtrup et al. 2000). This parasite is endemic, but non-pathogenic, in mice (*P. leucopus*) populations and vectored by the Ixodid tick, *I. scapularis* (also referred to as *Ixodes dammini*) in the northeastern United States (Gorenflot et al. 1998). In 1968, Scholtens et al. documented the first human case of *B. microti*. Since then, hundreds of cases have been reported, with approximately 40 resulting from blood transfusions and transplacental transmission (Lux et al. 2003; Mylonakis 2001). Most of the cases are tick-transmitted and occur in spleen-intact individuals. Humans with *B. microti* infections commonly exhibit a flu-like presentation including fever, malaise and myalgia, but they have also been reported to be asymptomatic (Gorenflot et al. 1998).

Babesia microti differs from the true *Babesias* by the existence of a pre-erythrocytic stage, during which the parasite enters mammalian host lymphocytes (Mehlhorn and Schein1984). This species is not transovarially transmitted through the tick vector, which

is characteristic of the *Babesia* sp. sensu stricto as discussed above, but is transstadially transmitted instead (Levine 1985). Morphologically this organism is classified as pleomorphic and can be seen predominantly as rings, but also as pairs and tetrads, in vertebrate blood. The tetrad formation of *B. microti* is distinct form that of *B. divergens* in that it is the result of the quaternary budding of one organism into four rather than the binary fission of one organism into two as seen with *B. divergens* (Levine 1985).

In addition to the classic cases of human babesiosis, a number of cases due to previously un-described *Babesia* species have been reported in recent years (Quick et al. 1993; Persing et al. 1995; Herwaldt et al. 1996, 2003, 2004; Beattie et al. 2002). The causative agents in three of these cases, despite occurring in North America, have been classified as *B. divergens*-like due to morphologic, antigenic, and small subunit ribosomal RNA (SSU rRNA) gene similarities (Herwaldt et al. 1996; Beattie et al. 2002; Herwaldt et al. 2003). The first (designated MO1) was diagnosed in an elderly asplenic Missouri man of failing health (Herwaldt et al. 1996). The patient presented febrile with headache, joint pain, thrombocytopenia and piroplasm-positive blood, despite having no recollection of a tick bite or history of foreign travel. Even with aggressive treatment including quinine sulfate and clindamycin, the patient suffered from organ failure and secondary infection and died twenty days post hospitalization.

Parasite morphology, serology, animal inoculation, and phylogenetic analysis were utilized to identify MO1 (Herwaldt et al 1996). Morphologically this organism is most similar to *B. divergens*, exhibiting subcentral and subperipheral pairs in human erythrocytes, which are consistent with human *B. divergens* infection, but not considered

diagnostic (Herwaldt et al. 1996). Serum taken from this patient was subjected to indirect immunofluorescent antibody and immuno-precipitation assays and reacted most strongly to *B. divergens* antigens. This serum reacted less strongly to antigens of the cervid parasite *B. odocoilei* and not at all to those of *B. microti*. In order to determine host infectivity of the parasite, animal inoculations were carried out using hamsters (*Mesocricetus auratus*), jirds (*Meriones unguiculatus*), asplenic calves, and bighorn sheep (*Ovis canadensis nelsoni*) (Herwaldt et al. 1996). Despite the fact that jirds and calves are susceptible to *B. divergens*, hamsters and jirds are susceptible to *B. microti* and the North American *Babesia* species designated WA1, and bighorn sheep are susceptible to *B. odocoilei*, none of the experimental animals became parasitemic after inoculation with the *B. divergens*-like MO1 organism (Herwaldt et al. 1996). Results of the phylogenetic analysis using partial SSU rRNA gene sequences show that there is a close relationship between the MO1 parasite and to both *B. divergens* and *B. odocoilei* (Herwaldt et al. 1996).

In 2002 Beattie et al. reported a second North American *B. divergens*-like case involving an asplenic Kentucky man with no history of European travel. The patient presented with headache, fever, and hemoglobinuria. Piroplasms were visualized in Giemsa-stained blood smears. Following diagnosis of babesiosis, intravenous clindamycin and oral quinidine therapy was initiated. Morphologically, as with MO1, this agent (designated KY for this discussion) most closely resembles *B. divergens*. SSU rRNA gene sequence analysis using the entire gene shows that the KY sequence is identical to that of MO1 and differs in only three nucleotide positions from that of *B. divergens* resulting in a 99.8% similarity between these two organisms (Beattie et al. 2002).

An epidemiologic survey on Nantucket Island found an organism described as *B. divergens* to be endemic in eastern cottontail rabbits (*Sylvilagus floridanus*) on the island (Goethert and Telford 2003a), which is designated NR for this discussion. The full length SSU rRNA gene sequence of NR is identical to those of both KY and MO1 (Goethert and Telford 2003a; Holman et al. 2005). Interestingly, Beattie et al. (2002) reported that the Kentucky patient had hunted and dressed cottontail rabbits prior to the onset of symptoms.

The most recent North American *B. divergens*-like case occurred in an elderly, asplenic, but otherwise healthy man living in Washington State (Herwaldt et al. 2004). The symptoms included fever, fatigue, and thrombocytopenia. After piroplasms were visualized by blood smear examination, he was diagnosed with babesiosis and treated accordingly. Despite temporary renal insufficiency resulting from dehydration, the patient recovered. As with the MO1 isolate, this organism had a strong serologic reactivity with *B. divergens*, but did not produce parasitemias when injected into hamsters or jirds. The full length SSU rRNA gene sequence varied in eight nucleotide positions from the European *B. divergens* sequence, resulting in a 99.5% similarity between these two organisms, and 99.6% similarity with the North American MO-1 and KY agents (Herwaldt et al. 2004; unpublished data).

A few agents of human babesiosis have been classified as distinct from both *B. microti* and *B. divergens*. The first was designated WA1 in reference to Washington State where it occurred in a relatively healthy man (Quick et al. 1993). The WA1 agent is morphologically and serologically most similar to the canine parasite *Babesia gibsoni,*

but failed to infect splenectomized dogs upon experimental inoculation. Four additional west coast cases of human babesiosis have occurred in California, and the causative agents appear to be conspecific with WA1 (Persing et al. 1995).

The EU1 (European Union 1) agent responsible for two cases of human babesiosis in Europe was found to most closely resemble *B. odocoilei* according to phylogenetic analysis, but still has 29 base differences in the SSU rRNA gene sequence comparison by 29 base differences (Herwaldt et al. 2004). The EU1 agent and *B. divergens* differ at 31 positions within the SSU rRNA gene sequence. Serologic, animal inoculation, and phylogenetic analysis distinguish this organism from other known human *Babesia* agents (Herwaldt et al. 2004).

Numerous techniques have been utilized for the identification of *Babesia* species. Initial morphologic examination using a Giemsa-stained blood smear is critical to the confirmation of patent babesiosis and may be conclusive in the presence of species specific diagnostic forms, such as the accolé form of *B. divergens* and the tetrad form of *B. microti,* when seen in the natural vertebrate host. Unfortunately, there may be no visible parasites, the vertebrate host may be aberrant or these forms may be absent in the sample smear making definitive identification through microscopy impossible (Herwaldt et al. 1996).

Serologic analyses have been useful tools in the characterization of numerous organisms, including *Babesia* (Waldrup et al. 1992; Goff et al. 1993; Holman et al. 2000). These methods, however, provide information about exposure to a species rather than indication of current infection (Langton et al. 2003; Gorenflot et al. 1998). Furthermore,

complications, such as false positives due to cross-reactivity, reduce the dependability of these analyses (Holman et al. 2000; Homer et al. 2000; Persing et al. 1995). The immune status of the patient may also alter serological results due to cross-reactivity resulting from connective tissue disorders or false negatives in immune compromised individuals (Benezra et al. 1987).

Generally speaking, *Babesia* are relatively host specific with regards to vertebrate host, pathogenicity, and tick vector (Levine 1985; Holman et al. 2000). Although these parasites are continually identified in a greater variety of host species than once thought, this trait has been utilized to differentiate between isolates through comparative in vivo inoculation studies (Herwaldt et al. 2000; Quick et al. 1993; Holman et al. 2005). In order to identify the MO1 agent, Herwaldt et al. (1996) inoculated animals known to be susceptible to specific *Babesia* species. No parasitemias resulted, distinguishing MO1 from *B. divergens*, *B. odocoilei*, *B. microti,* and WA1. The agent responsible for the WA1 babesiosis was also subjected to in vivo studies in which this organism was injected into hamsters, jirds and a dog. The hamsters and jirds became parasitemic, but the dog did not. These results suggest that although the organism appears most similar to *B. gibsoni*, its inability to infect the dog and its infectivity for rodents distinguishes it from this canine parasite.

The increased success of in vitro cultivation of *Babesia* species has created an additional diagnostic tool when blood smear results are inconclusive. *Babesia divergens*, *B. bovis*, *B. equi* and *Babesia caballi* and are among the commonly cultured *Babesia* species (Levy and Ristic 1980; Varynen and Tuomi 1982; Holman et al. 1993a, 1994b). This technique

provides a dependable source of parasites for investigations of resistance, treatment options, and genetic relationships, and has also proven to be a useful diagnostic tool (Holman et al. 1998; Sunaga et al. 2002; Malandrin et al. 2004). Holman et al. (1998) successfully utilized the microcentrifuge in vitro method to amplify *B. equi* parasitemias from naturally infected horses with low circulating parasitemias. This technique can therefore be applied to assess carrier status of animals as well as provide a sufficient number of organisms for use in comparative studies. In vitro culture was also used to assess the carrier status of cattle with *B. divergens* (Malandrin et al. 2004). It was found that this organism could be successfully cultured from asymptomatic carrier cattle when no parasites could be visualized by blood smear.

In vitro methods of *Babesia* cultivation were introduced following the success with the closely related parasite genus, *Plasmodium*. A constant flow system was implemented in the cultivation of *Plasmodium* species that consists of a settled erythrocyte layer over which the medium continually flows (Trager 1971). Through this method, greater parasite growth was found in a reduced oxygen environment (1% oxygen) relative to a gas mixture with higher oxygen content (5%) (Trager and Jenson 1976). A similar result was achieved with the candle jar method, which utilizes a lit candle to consume the oxygen, creating a reduced oxygen environment for the culture (Emerson and Held 1969; Trager and Jenson 1976).

Levy and Ristic (1980) developed the microaerophilous stationary phase (MASP) technique, which also involves a settled layer of erythrocytes and reduced oxygen tension, to successfully cultivate *B. bovis*. This system uses a particular depth of medium

to act as an oxygen barrier creating reduced oxygen tension resulting from parasite metabolism (Levy and Ristic 1980). As the oxygen tension of the erythrocyte layer declines, the culture turns noticeably darker. This color change has been correlated to parasite growth (Levy and Ristic 1980). Currently the MASP technique is routinely used for the in vitro culture of multiple *Babesia* species including *B. divergens*, *B. bigemina*, *B. caballi*, *B. equi*, *B. odocoilei* and *B. gibsoni* (Varynen and Tuomi 1982; Vega et al. 1985; Holman et al. 1998, 1993a, 1994b; Zweygarth and Lopez-Rebollar 2000).

Holman et al. (2005) successfully cultivated two isolates of the *B. divergens*-like agent (NR) from blood samples collected from eastern cottontail rabbits on Nantucket Island, Massachusetts. Both eastern cottontail rabbit and human erythrocytes supported continuous growth of both isolates. Of the numerous culture media tested, a supplemented HL-1 medium was found to be the best for long-term propagation. Serum comparisons found that the medium supplemented with human serum supported growth. However, parasite growth was not supported by media supplemented with fetal bovine or domestic rabbit sera.

Genetic sequence comparison analyses provide organism specific characterization. Restriction fragment length polymorphism (RFLP) and nucleotide sequence determination are commonly used polymerase chain reaction (PCR) based approaches of genetic sequence investigation that are useful for the characterization of organisms (Prichard and Tait 2001). The SSU rRNA gene produces a functionally conserved product, and therefore has been the gold standard in genetic analysis for Apicomplexan species (Prichard and Tait 2001; Reddy et al. 1991; Holman et al. 2000, Holman et al.

2003). Additionally, multiple copies of the SSU rRNA gene are present in most eukaryotic genomes, providing a sufficient amount of target DNA for amplification reactions, further making it an ideal target for genetic studies (Reddy et al. 1991). Through Southern blot analysis of digested rDNA, Reddy et al. (1991) identified three distinct rRNA transcriptional units in the genome of the cattle piroplasm *B. bigemina*. These nucleotide units varied in length from 10.65 kb and 10.80 kb to 13.65 kb. This result is supported by the earlier finding by Dalrymple (1990) that three distinct SSU rRNA transcriptional units exist in the genome of *B. bovis*.

Holman et al. (2000) utilized SSU rRNA gene sequence analysis to characterize suspected *B. odocoilei* isolates with identical clinical presentations but serologic variability. The SSU rRNA gene sequences of these cervid parasites were all identical to that of *B. odocoilei*. The majority of the SSU rRNA gene was also used by Goethert and Telford (2003b) to investigate the heterogeneity within the zoonotic rodent parasite *B. microti*. The authors divided the species into three distinct clades based on genetic variation, of which only two clades were found to be parasites of rodents. The remaining clade consisted of parasites of carnivores (Goethert and Telford 2003b).

The highly conserved nature of the SSU rRNA sequences does not allow for definitive characterization of the current *B. divergens*-like organisms (Herwaldt et al. 2000; Beattie et al. 2002; Langton et al. 2003). As seen with the MO1 and KY agents and the most recent agent of human babesiosis in Washington State, slight sequence variation exists among the SSU rRNA gene sequences of these organisms and that of *B. divergens*. Therefore, a definitive species assignment of *B. divergens* cannot be made for the agents

of these recent U. S. cases. This is also the situation with a *B. divergens*-like agent collected from reindeer in Great Britain with an SSU rRNA gene sequence that differs from that of *B. divergens* by only four nucleotide positions (Langton et al. 2003). The amount of SSU rRNA gene nucleotide variation required for species differentiation has not been defined. Within such a highly conserved gene no variation is expected, as seen among sequences acquired from multiple geographically distant isolates of *B. divergens* (Herwaldt et al. 2003). Despite this finding, it is likely that multiple SSU rRNA transcription units are present in the *B. divergens* genome, as reported for *B. bigemina* and *B. bovis*. The existence of multiple SSU rRNA units could justify the slight intra-species variation seen between these *B. divergens*-like isolates and *B. divergens* and allow the suggestion that some sequence variation may be possible within a species.

The ß-tubulin gene has also been utilized as a genetic marker for piroplasm species (Cacciò et al. 1997; Goethert and Telford 2003a; Goethert and Telford 2003b). This gene encodes for the functionally conserved component of eukaryotic cell microtubules, ß-tubulin, and, therefore, has a nucleotide sequence that is conserved within the phylum Apicomplexa. The existence of at least one intron within this gene has allowed species discrimination due to PCR product length variation. Cacciò et al. (1997) found ß-tubulin gene intron lengths in two species of *Theileria* and seven species of *Babesia* to vary from 20 to 170 bp. This variation allowed for species determination by PCR product size comparison in most cases. An RFLP protocol was developed to differentiate among those that could not be distinguished by PCR product size alone (Cacciò et al. 1997).

The ß-tubulin gene sequence has also been used to support *Babesia* SSU rRNA gene data. Goethert and Telford (2003b) sequenced the ß-tubulin gene of *B. microti* to investigate the genetic diversity of this organism, which appears to be underestimated with the highly conserved SSU rRNA gene. The majority of human *B. microti* infections have occurred in the northeastern United States (Goethert and Telford 2003b). Despite the existence of both a competent rodent host and tick vector (*I. ricinus*), only a few cases of *B. microti* babesiosis in man have been reported in Europe (Goethert and Telford 2003b; Duh et al. 2001; Grey et al. 2002). This evidence, in addition to inconsistent *B. microti* infectivity in Japan, suggests that different strains of *B. microti* exist (Goethert and Telford 2003b; Saito-Ito et al. 2004). Genetic diversity was found among morphologically invariant *B. microti* isolates. The phylogenetic separations resulting from the SSU rRNA gene analyses were supported by the ß-tubulin gene data, which had more distinct sequence variation (Goethert and Telford 2003b). In order to further identify the *B. divergens*-like organism found in cottontail rabbits on Nantucket Island, Goethert and Telford (2003a) used ß-tubulin gene sequence data in addition to SSU rRNA gene data. This less highly conserved gene sequence supports the evidence that this organism is most closely related to *B. divergens*.

Other less highly conserved genetic regions have also been investigated for their use in the distinction between closely related organisms and to delineate species within a genus (Berzunza-Cruz et al. 2002). The ribosomal RNA (rRNA) internal transcribed spacers (ITS1 and ITS2) are non-translated genetic regions that lie between the highly conserved SSU rRNA (5´) and LSU rRNA (3´) genes and are separated by the smaller 5.8S rRNA gene in most eukaryotic organisms (Berzunza-Cruz et al. 2002). The rRNA ITS1 and

ITS2 genetic region sequence comparisons have reinforced the distinction between subspecies of *Babesia canis* isolates previously suggested according to pathogenicity, vector specificity, and geography (Uilenberg et al. 1989; Zahler et al. 1998). The rRNA ITS genetic region sequences of the three previously designated subspecies, *Babesia canis canis*, *Babesia canis vogeli* and *Babesia canis rossi* shared 99.0-97.9 percent identity within subspecies and 69-82 percent identity among subspecies. The rRNA ITS genetic region sequences of the *B. canis* subspecies shared only 66-69 percent identity with the SSU rRNA ITS genetic region of *B. caballi,* an equine parasite (Zahler et al. 1998). The authors concluded that, independent of environmental factors, the three suggested subspecies of *B. canis* can be delineated genetically. Furthermore, the *B. canis* group can be differentiated from other distinct *Babesia* species using rRNA ITS genetic region comparisons.

Additional Apicomplexan parasites have been evaluated according to rRNA ITS1-5.8S-ITS2 genetic region sequences. Collins and Allsopp (1999) found that the 5.8S rRNA gene sequences of eleven *Theileria* isolates are identical, but the rRNA ITS1 and ITS2 genetic region sequences show high variability. The variable combinations found within the rRNA ITS1 and ITS2 genetic region sequences of *Theileria parva parva* and *Theileria parva lawrenci* suggest that genetic recombination occurs between these two organisms, creating more variability within these already poorly conserved sequences (Collins and Allsopp 1999). Adam et al. (2000) also used rRNA ITS1 genetic region sequences to investigate variability within the intestinal Apicomplexan parasite species *Cyclospora cayetanensis* in order to identify distinct genotypes of this organism.

Sequence heterogeneity was found within isolates suggesting that there may be multiple rRNA transcriptional units within the genome of this organism.

The goals of this project include determining the usefulness of the rRNA ITS1-5.8S-ITS2 genetic region in the identification of the *B. divergens*-like parasites NR, KY, and GBRD, the Great Britain reindeer *B. divergens*-like agent. Also, baseline data obtained from this genetic region may be used in the characterization of other *Babesia* species. Following the success of Zahler et al. (1998) with *B. canis*, it is expected that this region will be helpful in the characterization of these agents.

In vitro cell culture comparisons will also be employed to determine if the *B. divergens*-like organism from eastern cottontail rabbits and *B. divergens* share the same host erythrocyte requirements. This technique parallels in vivo studies in which parasites are inoculated into hosts with known susceptibilities as carried out by Quick et al. (1993) and Herwaldt et al. (1996).

MATERIALS AND METHODS

rRNA ITS1-5.8S-ITS2 Genetic Sequence Comparisons

Parasites and DNA extraction

Six *Babesia* isolates were included in this study: three previously described *B. divergens*-like parasites from a human case of babesiosis in Kentucky (KY), from an eastern cottontail rabbit on Nantucket Island, Massachusetts (NR), a reindeer from Great Britain (GBRD) (Beattie et al. 2002; Goethert and Telford 2003a; Langton et al. 2003); a *B. divergens* (BdivP; Purnell isolate of cattle origin from Great Britain) (Purnell et al. 1976; Holman et al. 2000); and two strains of *B. microti*, Peabody and GI, of human origin (Ruebush and Hanson 1979; Peisman et al. 1986) (Table 1).

Parasite DNA was obtained from the Kentucky human isolate and the Great Britain reindeer as previously reported (Beattie et al. 2002; Langton et al. 2003). Genomic DNA of NR was extracted from infected eastern cottontail rabbit blood collected into acid citrate dextrose (ACD) during an epidemiologic survey on Nantucket Island, Massachusetts (Goethert and Telford 2003A). *Babesia microti* DNA was extracted from blood of mice experimentally infected with either the GI or Peabody *B. microti* strains. *Babesia divergens* DNA was obtained from parasites recovered from cryopreserved *B. divergens* culture stocks and cultivated as previously described except for the use of HL-1 medium (BIO Whitaker Walkersville, MD) supplemented with 20%

Table 1. List of taxa from which rRNA ITS1-5.8S-ITS2 genetic region sequence data included in the analyses were derived. GenBank accession numbers for the sequences are provided. Sequence data generated in this study are indicated by an asterisk (*).

Isolate	Origin	Host of origin	Taxon	GenBank accession number
KY	Kentucky	Human	*Babesia divergens*-like	*
NR	Nantucket Island, Massachusetts,	Cottontail rabbit	*B. divergens*-like	*
GBRD	Great Britain	Reindeer	*B. divergens*-like	*
BdivP	Great Britain	Cow	*B. divergens* (Purnell)	*
MNBo	Minnesota	Caribou	*Babesia odocoilei*	AY339756 (Clone 1)
				AY339757 (Clone 2)
				AY339758 (Clone 3)
WIBo	Wisconsin	Reindeer	*B. odocoilei*	AY339749 (Clone 1)
				AY339750 (Clone 2)
				AY345122 (Clone 3)
GIBm	Boston, Massachusetts	Human	*Babesia microti* (GI)	*
PeaBm	Nantucket Island, Massachusetts	Human	*B. microti* (Peabody)	*
BcanR	South Africa	Dog	*Babesia canis*	AF394535
BcanV	Spain	Dog	*B. canis*	AF394534
BcanC	Europe	Dog	*B. canis*	AF394533
BcabN	Namibia	Horse	*Babesia caballi*	AF394536

adult bovine serum (Holman et al. 2000). Parasite DNA was purified from the cultures when approximately 10% parasitemia was achieved. In all cases, parasite DNA was purified from infected erythrocytes by a standard phenol/chloroform extraction protocol using phase lock divider gel tubes (Eppendorf, Westbury, NY) (Sambrook et al. 1989).

rRNA ITS1-5.8S-ITS2 genetic region amplification and sequencing

The rRNA ITS1-5.8S-ITS2 region was amplified by a nested polymerase chain reaction (PCR). The primers used to amplify the target gene region in this study were designed previously for use in this lab. A map of the rRNA ITS genetic region with the relative locations of the following primer sequences can be seen in Fig 1. The primary PCR reactions (Advantage 2 PCR Enzyme System, CLONTECH Laboratories, Palo Alto, CA) were performed in a 25 µl reaction volume with 50 -100 ng DNA and one of the following primer combinations (0.1 µM of each primer): ITSF (5' GAGAAGTCGTAACAAGGTTTCCG 3') and ITSR (5' GGTCCGTGTTCCAAGACGG 3') (BdivP, KY, GBRD), ITSF and LSU50R (5' GCTTCACTCGCCGTTACTAGG 3') (PeaBm), or 1055F (5' GGTGGTGCATGGCCG 3') and ITSR (NR and GIBm). Amplification was carried out in a thermal cycler (Express or Sprint Thermal Cycler; Hybaid, Middlesex, United Kingdom) using a hot start method. Initial denaturation for 1-5 min at 94-96 ºC was followed by 35 cycles of denaturation for 30 sec at 94 ºC, annealing for 30 sec at 55 ºC, and extension for 2 min at 72 ºC. A final extension of 10 min at 72 ºC was followed by a 4 ºC hold. All PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized by UV transillumination.

Nested protocols to obtain single band amplicons for KY, NR, GBRD, and GIBm, used 1 µl of the primary PCR product as template in amplifications performed as above, but with primer combinations ITSF and LSU50R (KY, GBRD, and GIBm) or ITSFN (5' GTGAACCTGCGGA-AG 3') and LSU50R (NR).

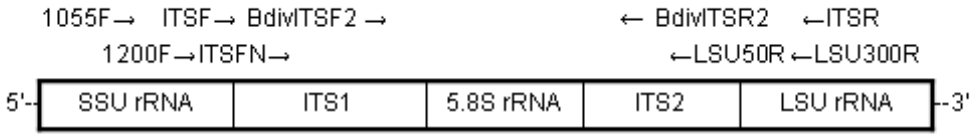


Figure 1. Schematic drawing of the rRNA ITS1-5.8S-ITS2 genetic region. The primers used to amplify the rRNA ITS1-5.8S-ITS2 genetic region are shown with arrows indicating primer orientation.

Except for the PeaBm amplicon, which was PCR purified (QIAquick; Qiagen, MD) and directly sequenced using primers ITSFN and LSU50R, the final amplicons were cloned prior to sequencing following manufacturer's instructions (TOPO One Shot; Invitrogen, San Diego, CA). Color-selected transformed colonies were screened by colony PCR using plasmid promoter region primers m13 forward and m13 reverse. Colony PCR reactions were carried out using a sample of each chosen colony mixed with 9 µl of water and incubated at 94 ºC prior to the addition of primers (0.1 µM each) and polymerase and

buffer solution (Taq PCR master mix, Qiagen, Valencia, CA) in a final reaction volume of 22 µl. Following the 10 minute denaturation, the amplification protocol included 30 cycles of 94 ºC for 1 min, annealing at 50 ºC for 1 min and a 1 min extension at 72 ºC. A final extension at 72 ºC for 10 minutes was followed by a 4 ºC hold. Colony PCR products were electrophoresed on 1% agarose gels, stained with ethidium bromide and visualized by UV transillumination. Plasmid DNA preparations were performed on positive colonies using the QIAprep Spin Miniprep kit following manufacturer's instructions (Qiagen). Three clones for each isolate were sequenced in both directions using plasmid promoter region specific primers, m13 reverse and m13 forward, and specific SSU rRNA ITS genetic region primers as needed (ITSF, ITSFN, ITSR, LSU50R, 1200F (5' CAGGTCTGTGATGCT 3'), BdivITSF2 (5' CACGGATGCTGCTCG 3'), LSU300R (5' TWGCGCTTCAATCCC 3'), and BdivITSR2 (5' CCGGTAGCATGCTATGG 3')). The cloned amplicons were sequenced by the Gene Technologies Laboratory (Institute of Developmental and Molecular Biology, Department of Biology, Texas A&M University, College Station, TX) or the DNA Technologies Core Lab (Department of Veterinary Pathobiology, Texas A&M University).

Sequence analysis

Sequencher (3.1.2) software (Gene Codes Corp., Ann Arbor, MI) was used to construct contiguous rRNA ITS1-5.8S-ITS2 genetic region sequences for each KY, NR, BdivP, GBRD and GIBm clone, and for the directly sequenced PeaBm. Intra-isolate pair wise comparisons among three clones for each isolate were obtained for the rRNA ITS1, 5.8S

and ITS2 genetic regions separately using Genestream (Pearson et al. 1997). Corresponding values were obtained for two *B. odocoilei* isolates (MNBo and WIBo) using sequence data in the GenBank database (Table 1) (Holman et al. 2003). Consensus rRNA ITS1-5.8S-ITS2 genetic region sequences were determined from three clones of each isolate, including MNBo and WIBo, using Sequencher software. Intra- and intergenic pair wise alignments of the rRNA ITS genetic region consensus sequence data were performed using Genestream (Pearson et al. 1997).

Sequence data for the 5.8S rRNA genes from *Babesia canis rossi* (BcanR), *Babesia canis vogeli* (BcanV), *Babesia canis canis* (BcanC) and *Babesia caballi* (BcabN) obtained from the GenBank database (accession numbers AF394535, AF394534, AF394533 and AF394536, respectively) (Zahler et al. 1998) were used to determine intra- and intergenic percent identity values for the 5.8S rRNA gene sequences using Genestream pair wise comparisons.

Phylogenetic analyses

Three separate sequence alignments, consisting of the rRNA ITS1 and ITS2 genetic regions and the full rRNA ITS1-5.8S-ITS2 genetic region were constructed from three clones each of NR, KY, GBRD, BdivP, MNBo and WIBo, with the GIBm and PeaBm sequences included as outgroups**,** using Clustal W (Pearson et al. 1997) and manually adjusted as needed to maximize identity. Maximum likelihood phylogenetic trees employing a heuristic search strategy with general search options (bootstrap, n=100) were constructed from each resulting alignment using the Paup 4.0b10 program (Swofford, 2002). Likelihood analyses were carried out through a substitution model (Ti/Tv

ratio=1). The sequences of the ingroup were rooted against those of the outgroup at the internal node with basal polytomy. Groups with frequencies greater than 50% were retained and only the best trees were kept.

In Vitro Culture Comparisons

Parasites

Both the *Babesia* sp. isolated from Nantucket Island cottontail rabbit number 774 (NR) and the Purnell isolate of *B. divergens* were previously described (Goethert and Telford 2003a; Holman et al. 2005; Purnell et al. 1976). Cultured parasites retrieved from cryopreserved stocks were used in this study (Holman et al. 2000, 2005).

Parasite culture

Parasites were recovered from liquid nitrogen storage using previously described methods (Holman et al. 1993b). Donor erythrocytes for culture were prepared for use as previously described (Holman et al. 2005) and added to culture wells at a final well concentration of 10% (V/V) in the cultures. Each well contained 0.9 ml medium, 0.1 ml uninfected erythrocytes and 0.25 ml of the infected erythrocyte sample. This volume of 1.25 ml per well was maintained throughout the experiment. All cultures were incubated in a humidified modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, CA) at 37 ºC in a 5% carbon dioxide, 2% oxygen and 93% nitrogen atmosphere for the duration of the study.

Passages 14 and 23 of NR were recovered from cryostorage into cottontail rabbit donor erythrocytes and human donor erythrocytes (Rockland Immunochemicals, Gilbertsville, PA), respectively. The donor cottontail rabbit blood was collected into vacutainer tubes containing acid citrate dextrose from free-ranging eastern cottontail rabbits live-trapped under a scientific collecting permit issued by the Massachusetts Division of Fisheries and Wildlife by Drs. Heidi Goethert and Sam Telford, III (Division of Infectious Diseases, Department of Biomedical Sciences, Tufts University School of Veterinary Medicine, 200 Westboro Road, North Grafton, Massachusetts). Blood samples were determined to be free of *B. divergens* and the rabbit *Babesia* sp. by a previously described PCR performed by Dr. Heidi Goethert prior to shipping the blood to Texas A&M University (Goethert and Telford 2003a). Cultures of NR were maintained in complete culture medium, designated HL-H, comprised of HL-1 medium (Bio-Whittaker, Walkersville, MD) supplemented with 20% human serum (Rockland Immunochemicals), 1% HB101 (V/V, reconstituted according to manufacturer's instructions, Irvine Scientific, Santa Ana, CA), 200 mM sodium hypoxanthine and 32 µM thymidine (Holman et al. 1994b) (HT supplement, Gibco, Grand Island, NY), 1 mM L-glutamine (Gibco), antibiotic-antimycotic (200 µg/ml streptomycin, 200 U/ml penicillin, 50 µg/ml Fungizone, Gibco) and 100 µg/ml gentamycin (Gibco).

Passage two of *B. divergens* was recovered from cryostorage into bovine erythrocytes (adult crossbred, animal number 0425, Texas A&M University, College Station, TX) and maintained in HL-1 medium supplemented with L-glutamine, antibiotic-antimycotic, and gentamycin as above, but with 20% adult autologous bovine serum and designated

HL-B. Cryopreserved passage two *B. divergens* culture was also resuscitated into human erythrocytes and cultured in HL-H as described above for NR.

Medium was replenished daily in all cultures by removal of 0.9 ml of used medium, without disturbing the settled erythrocyte layer, and replacement with an equal volume of appropriate fresh medium. Initially, parasitemias in all cultures were monitored through Giemsa-stained (Accustain, Sigma) spots of erythrocytes as previously described (Holman et al. 2005). Subcultures were performed at a 1:2 split ratio every 3 to 7 days as the parasitemias exceeded 50 infected erythrocytes per spot. After parasitemias were sustained at approximately 2-3 % for two passages, the cultures were maintained on a 3-day 1:5 split ratio schedule for the remainder of the experiment. During 1:5 subculture, the used medium was removed and replaced with fresh. The settled erythrocyte layer was then resuspended and 0.25 ml of that resuspension was added to each of two wells containing 0.9 ml fresh medium and 0.1 ml uninfected erythrocytes.

The cultured NR and *B. divergens* were subcultured into the combinations of erythrocytes and media as shown in Table 2. Three passages, each at a 1:5 split ratio, were completed for both NR in bovine erythrocytes and *B. divergens* in cottontail erythrocytes prior to performing comparative growth assays to ensure that the original host erythrocytes were diluted out (Holman et al. 1993b).

Table 2. List of cultured parasites showing the different erythrocyte and media conditions.

Parasite	Host RBC	Medium
B. divergens	Human	HL-H
	Bovine	HL-H
	Bovine	HL-F
	Cottontail Rabbit	n/a
NR	Human	HL-H
	Human	HL-F
	Bovine	n/a
	Cottontail Rabbit	HL-H

In addition, each parasite was cultured as above, but using HL-1 medium supplemented with 20% fetal bovine serum (Atlanta Biologicals) and L-glutamine, antibiotics and gentamicin, designated HL-F, to evaluate the ability of fetal bovine serum to support continuous cultures (Table 2). Three passages were completed for both parasites in HL-F prior to beginning the experimental period. *Babesia divergens* in bovine erythrocytes and NR in human erythrocytes were subcultured into the HL-F medium and maintained following a normal 3-day subculture routine for 30 days as described above.

Evaluation of in vitro proliferation

Two wells of each culture combination were maintained and monitored for 30 days (10 passages). Four erythrocyte smears were made daily from each distinct culture (two smears per well), stained with Giemsa, and viewed at 1000X under oil immersion. Daily percent parasitized erythrocytes (PPE) were assessed by differentially counting 1,000 erythrocytes per smear in the four smears of each culture condition daily for the 30-day experimental period. The four PPE values obtained daily over the 30-day period were

averaged for each culture condition, and the Student's *t*-test used to determine significance between culture conditions.

The length and width of each individual parasite in 50 paired intraerythrocytic merozoites were measured for *B. divergens* and NR in each culture condition using an ocular micrometer at 1000X magnification under oil immersion. The average sizes and standard deviations were obtained for each *Babesia* sp. in each host erythrocyte type, and the significance of differences between them were assessed using Student's *t*-test.

RESULTS

rRNA ITS1-5.8S-ITS2 Genetic Sequence Comparisons

Sequence comparison

The rRNA ITS1-5.8S-ITS2 genomic DNA segments obtained from the six *Babesia* samples ranged in length from 708 base pairs (bp) in *B. microti* to 865 bp in the *B. divergens*-like NR and KY isolates (Table 3). NR and KY had sequences of identical length in all three regions, as did the two *B. microti* strains, GIBm and PeaBm (Table 3). The lengths of the rRNA ITS1 and ITS2 genetic regions were variable among the remaining isolates and within the GBRD and *B. odocoilei* isolates (Table 3). The 5.8S genes were 159 bp in length in *B. divergens* and in all *B. divergens*-like sequences, including *B. odocoilei* (Table 3).

Intra-isolate variation from 0 to 4 bp within the rRNA ITS1 genetic region, 5.8S rRNA gene or rRNA ITS2 genetic region sequences occurred within NR, KY and BdivP, with variation in the full rRNA ITS1-5.8S-ITS2 genetic region ranging from 0 to 7 bp among clones (Table 4). The rRNA ITS1, 5.8S rRNA or SSU rRNA ITS2 genetic region sequences of GBRD varied from 0 to 16 bp, with variation of 7 to 20 base pairs in the full rRNA ITS1-5.8S-ITS2 genetic region. MNBo and WisBo differed by as many as 29 bp in the ITS1 region and 38 bp in the full rRNA ITS1-5.8S-ITS2 genetic region (Table 4). The three clones of GIBm shared identical sequences (Table 4). Corresponding percent identity values to the above base pair differences are shown in Table 4. No

clones were available for analysis for PeaBm, which was sequenced directly from purified PCR product.

A full alignment of the rRNA ITS1-5.8S-ITS2 genetic region consensus sequences of NR, KY, BdivP and GBRD shows that the NR and KY sequences are identical (depicted as NR/KY) (Fig. 2). The BdivP and GBRD sequences were identical in 20 positions that were distinct from NR/KY (Fig. 2). The sequences of BdivP and NR/KY were the most divergent, with different nucleotides found in 54 positions. The GBRD sequence differed at 50 positions from BdivP and at 39 positions from NR/KY.

Table 3. List of rRNA ITS1-5.8S-ITS2 genetic region sequence lengths. Sequence lengths in base pairs of the SSU rRNA ITS1, 5.8S and ITS2 genetic regions from *Babesia divergens* (BdivP); *Babesia divergens*-like isolates from Kentucky human (KY), Nantucket Island rabbit (NR), and Great Britain reindeer (GBRD); *Babesia odocoilei* from Minnesota caribou (MnBo) and Wisconsin reindeer (WIBo), and *Babesia microti* strains GI (GIBm) and Peabody (PeaBm).

		Lengths of regions in base pairs		
	Isolate	ITS1	5.8S	ITS2
KY	Clone 1,2,3	449	159	257
NR	Clone 1,2,3	449	159	257
BdivP	Clone 1,2,3	446	159	246
GBRD	Clone 1,2	446	159	251
	Clone 3	448	159	251
MNBo	Clone 1,2	418	159	251
	Clone 3	416	159	251
WIBo	Clone 1	417	159	250
	Clone 2,3	413	159	250
Pea Bm		353	156	197
GI Bm	Clone 1,2,3	353	156	197

Table 4. Table of clonal sequence comparisons. Pairwise sequence comparisons of the rRNA ITS1 genetic region, 5.8S rRNA gene and rRNA ITS2 genetic region among clones of *Babesia divergens* (BdivP); *Babesia divergens*-like isolates from Kentucky human (KY), Nantucket Island rabbit (NR), and Great Britain reindeer (GBRD); *Babesia odocoilei* from Minnesota caribou (MnBo) and Wisconsin reindeer (WIBo), and *Babesia microti* GI strain (GIBm). The values in each upper matrix represent base pair differences. The values in each lower matrix represent percent identities.

Isolate	Clone 1			Clone 2			Clone 3		
	ITS1	5.8S	ITS2	ITS1	5.8S	ITS2	ITS1	5.8S	ITS2
KY clone 1	-	-	-	1	1	0	1	3	0
KY clone 2	99.8	99.4	100	-	-	-	2	2	0
KY clone 3	99.8	98.1	100	99.6	98.7	100	-	-	-
NR clone 1	-	-	-	2	2	3	2	1	3
NR clone 2	99.6	98.7	98.8	-	-	-	0	1	4
NR clone 3	99.6	99.4	98.8	100	99.4	99.2	-	-	-
BdivP clone 1	-	-	-	2	0	2	0	0	0
BdivP clone 2	99.6	100	99.2	-	-	-	2	0	2
BdivP clone 3	100	100	100	99.6	100	99.2	-	-	-
GBRD clone 1	-	-	-	2	1	4	16	0	1
GBRD clone 2	99.6	99.4	98.5	-	-	-	14	1	5
GBRD clone 3	96.4	100	99.6	96.9	99.4	98.1	-	-	-
MNBo clone 1	-	-	-	2	1	0	14	0	1
MNBo clone 2	99.5	99.4	100	-	-	-	14	2	0
MNBo clone 3	96.7	98.1	100	96.7	98.7	100	-	-	-
WIBo clone 1	-	-	-	29	0	9	28	0	10
WIBo clone 2	93.1	100	96.4	-	-	-	1	0	1
WIBo clone 3	93.3	100	96.0	99.8	100	99.6	-	-	-
GIBm clone 1	-	-	-	0	0	0	0	0	0
GIBm clone 2	100	100	100	-	-	-	0	0	0
GIBm clone 3	100	100	100	100	100	100	-	-	-

```
NR/KY     ACATTGAATACCCTTGCACAATAGTGCTCGGCTTCGACATTTACGTTGTGTAAGCTTGCT 60
GBRD      ACATTGAATACCTTGCACAATAGTGCTCGGCTTCGACATTTACGTTGTGTAAGCTTGCT
BdivP     ACATTGAATACCCTTGCACAATAGTGCTCGGCTTCGGCATTTACGTTGTGTAAGCTTGCT

NR/KY     TGCAGCTGTGACTCTACGTCATGGTCCACTTTCTGTGGTTTCGTATTTGCCGTTGCCATG 120
GBRD      TGCAGCTGTGACTCTACGTCATGGTCCACTTT--GTGGTTTCGTACTTGCCGTTGCCATG
BdivP     TGCAGCTGTGACTCTACGTCATGGTCCACTTTTGGTGGTTTCGTATTTGTCGTTGCCATG

NR/KY     GCGACGTGGTTTCGGTCTTGTTCCGTTTCCATCCCTGCGCTTTTGCGTGGGACGTTGCCC 180
GBRD      GCGACGTGGTTTCGGTCTTGTTCCGTTTCCATCCCTACGCTTTTGCGTGGGACGTTGCCC
BdivP     GCGACGTGGTTTCGGTCTTGTTCCGTTTCCATCCCTGCGCTTTTGCGTGGGACGTTGCCC

NR/KY     CCTCCCACCCCACCGGGTGTATACTCCACTACGGTATAACTACTGTAGATGATATACACCT 240
GBRD      CCTCCCACCCCACCGGGTGTATGTTTACTACGGTGTAACTACTGTAGTTGATATACACTT
BdivP     CCTCCCACCCCACCGGGTGTATGTTTACTGCGGTGTAACTACTGTAGTTGGCGTACACTT

NR/KY     GGGTTATGCTGTGTCGATACTGATGTTACTGTTGATTGCTCTTCGAGTAGTTGTTAGTAA 300
GBRD      GGGTTATGCTGTGTCAATACTGATGTTACTATTGAGTGCTCTTCGAGTAGTTGCTAGTAG
BdivP     GGGTTATGCCGTGTCGATACTGATGTTACTAGTGATTGCTCTTTGAGTGGTTGTTAGTGC

NR/KY     CTTACCGGTGTTGCCACGGATGCTGCTCGTGGATCTATTAGATTCAAGCAGTTGCTGCTT 360
GBRD      --TAGCAGTACTGTCACAGATGTTGCTCGTGGATCTATTAGATTCAAGCAGTTGCTGCTT
BdivP     --TA-CAGTGTTGTCACGGATGCTGCTCGTGGATCTAATAGATTCAAGCAGTTGCTGCTT

NR/KY     CGTGCAGTGTCTTGCGTAGCGATTTCGTTACGATAATGCAACTCCGCTCGTTCATCGTTT 420
GBRD      CGTGCAGTGTTTAGCGTAGCGATTTCGTTACGATAATGCAACTCCGCTCGTTCATCGTTT
BdivP     CGTGCAGTGTTTTGCGTAGCGATTTCGTTACGATAATGCAACTCCGCTCGTTCATCGTCT

NR/KY     T-GCGTTGTTCGAGTTTGTTTAGAAATTATAAACTTTCAGCGATGGATGTCTTGGCTCAC 480
GBRD      TTGCGTTGTTCGAGTTTGTTTAGAAATTATAAACTTTCAGCGATGGATGTCTTGGCTCAC
BdivP     T-GCGTTGTTCGAGTTTGTTTAGAAATTATAAACTTTCAGCGATGGATGTCTTGGCTCAC

NR/KY     ACAACGATGAAGGACGCAGCAAATTGCGATAAGCATTATGACTTGCAGACTTCTGCGATT 540
GBRD      ACAACGATGAAGGACGCAGCAAATTGCGATAAGCATTATGACTTGCAGACTTCTGCGATT
BdivP     ACAACGATGAAGGACGCAGCAAATTGCGATAAGCATTATGACTTGCAGACTTCTGCGATT

NR/KY     TAACAGACCTCTGAACGTAACAAACACACCGCCTCTGCTCGCATGCGGTACTCCCGTTTC 600
GBRD      TAACAGACCTCTGAACGTAACAAACACACCGCCTCTGCTCGCATGCGGTACTCCCGTTTC
BdivP     TAACAGACCTCTGAACGTAACAAACACACCGCCTCTGCTCGCATGCGGTACTCCCGTTTC

NR/KY     AGTGAGCCCCCTTTCCTAAAGGAACCACACTTTTACTGGTTTACTACTGGTATTTTGTTGT 660
GBRD      AGTGAGCCCCCTTTCCTAAAGGAACCACACTTTTACTGGTTTACTACTGGTATTT---GT
BdivP     AGTGAGCCCCCTTTCCTAAAGGAACCAGACTTTTACTC--------CTGGTAATA---GT

NR/KY     ATGGTCGGTCCTTTGCGAGTGGGTGTTGTGACAATCACCTTAATTTCCATAGCATGCTAC 720
GBRD      ATGGTCAGTCCTTTGCGAGTGGGTGTTGTGACAATCACCTTAATTTCCATAGCATGCTAC
BdivP     ATGGTTAGTCCTTTGCGAGTGGGTGTTGTGACAATCACCTTAATTTCCATAGCATGCTAC

NR/KY     CGGGTATCGCCACGTGTGATCTCGAAGCTTCTTGTTGTAATTTATTACTCTAGGCTTCTT 780
GBRD      CGGGTATCGCCACGTGTGATCTCGAAGCTTCTTGTTGTAATTTATTACTCTAGGCTTCTT
BdivP     CGGGTATCGCCACGTGTGATCTCGAAGCTTCTTGTTGTAATTTATTACTCTAGGCTTCTT

NR/KY     TTGAGATGTGCGACTAAGAATTGCTATTGTAGTATTTCTACAGCAAGTGGATGATGCTAG 840
GBRD      TTGAGATGTGCGGCTAAGAATTACTATTGTA---TTTCTATAGCAAGTGGATGATGCTAG
BdivP     TTGAGATGTGCGGCTAGGAATTACTATTGCAGTATTTCTATAGCAAGTGGATGATGCTAG

NR/KY     TGTTGTCAGTGCTATAAAATTTTTAT                                   900
GBRD      TGTTGTCAGTGCTATAAAATTTTAAT
BdivP     TGTTGTCAGTGCTATAAGTTTGAAT
```

Figure 2. Alignment of the rRNA ITS1-5.8S-ITS2 genetic region sequences. Clustal W aligned consensus sequences from the rRNA ITS1-5.8S-ITS2 genetic region of the *Babesia divergens*-like isolates from the Kentucky human and Nantucket Island rabbit (KY/NR), and from *Babesia divergens* Purnell (BdivP) and the Great Britain reindeer *Babesia divergens*-like parasite (GBRD). The rRNA ITS1 and ITS2 genetic regions sequences are shown in normal font. The intervening 5.8S rRNA gene sequences are italicized.

The KY and NR rRNA ITS1 and ITS2 genetic region sequences shared percent identities of 89.9 and 93.5 with those of BdivP, and 95.7 and 93.8 with those of GBRD, respectively (Table 5). The consensus sequences for the PeaBm and GIBm isolates shared 98.9 percent identity in the rRNA ITS1 genetic region and 99.5 percent identity in the rRNA ITS2 genetic region (Table 5). The *B. microti* rRNA ITS1 and ITS2 genetic region sequences shared percent identities ranging from 45.3 to 48.7 with those of the *B. divergens* and *B. divergens*-like parasites (Table 5). MNBo and WIBo rRNA ITS1 and ITS2 genetic regions consensus sequences shared 92.2 and 94.8 percent identity, respectively (Table 5). MNBo and WIBo shared percent identities ranging from 67.0 and 73.1 with NR, KY, BdivP and GBRD in both the rRNA ITS1 and ITS2 genetic regions (Table 5). *Babesia odocoilei* ranged from 45.3 to 47.3 percent identity with *B. microti* in both ITS1 and ITS2 regions (Table 5).

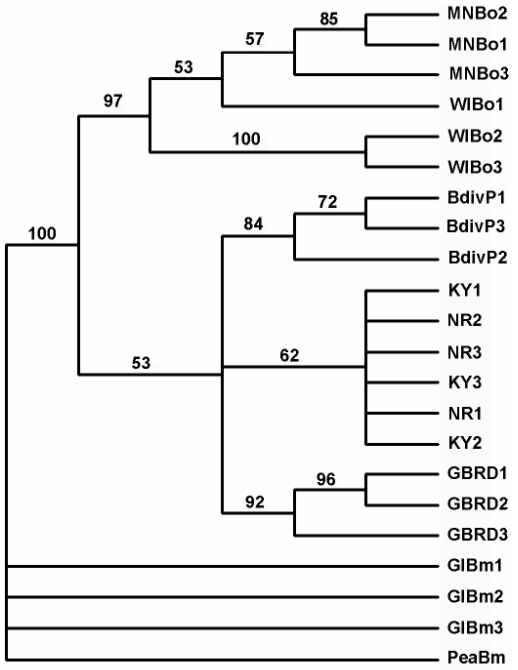
The 5.8S rRNA gene sequences for all isolates were identical except for those of *B. microti*. GIBm and PeaBm were identical to each other but shared only 78.6 percent identity with the other isolates (data not shown).

Analysis of the 5.8S rRNA gene sequence data for *B. canis rossi*, *B. canis vogeli*, *B. canis canis* and *B. caballi* from the GenBank database shows that the 5.8S rRNA gene sequence of BcanR shared 96.9 percent identity with those of both BcanV and BcanC. The BcanV sequence shared 97.5 percent identity with that of BcanC. The 5.8S rRNA genes of BcanR, BcanV and BcanC shared 95.6, 95.0 and 94.3 percent identity, respectively, to that of BcabN.

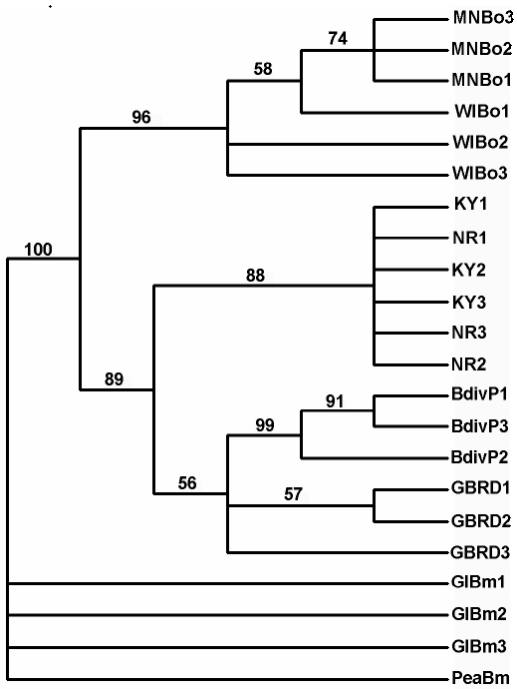
Table 5. List of consensus sequence percent identities. Percent identities found in the rRNA ITS1 and ITS2 regions consensus sequences of *Babesia divergens* (BdivP); *Babesia divergens*-like isolates from Kentucky human (KY), Nantucket Island rabbit (NR), and Great Britain reindeer (GBRD); *Babesia odocoilei* from Minnesota caribou (MnBo) and Wisconsin reindeer (WIBo), and *Babesia microti* strains GI (GIBm) and Peabody (PeaBm). The values in the upper matrix were calculated using the ITS1 sequences and the values in the lower matrix were calculated with the ITS2 sequences.

Isolate	KY	NR	BdivP	GBRD	MNBo	WIBo	PeaBm	GIBm
KY	-	100	93.5	93.8	67.8	68.7	45.6	45.3
NR	100	-	93.5	93.8	67.8	68.7	45.6	45.3
BdivP	89.9	89.9	-	93.5	69.0	68.7	45.9	45.5
GBRD	95.7	95.7	92.1	-	67.2	67.0	46.8	45.7
MNBo	73.1	73.1	70.4	72.5	-	9.2.	46.5	47.8
WIBo	71.3	71.3	71.0	72.9	94.8	-	48.4	47.2
PeaBm	46.8	46.8	48.2	48.5	47.4	48.7	-	98.9
GIBm	47.2	47.2	48.2	47.7	47.6	47.9	99.5	-

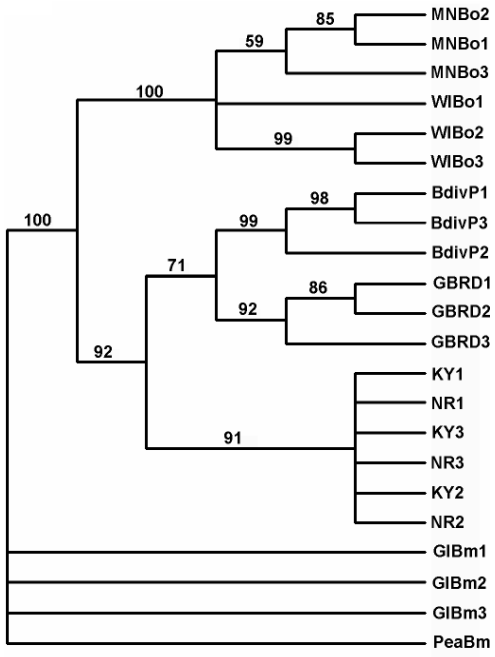
Phylogenetic trees were inferred from the rRNA ITS1, ITS2 and ITS1-5.8S-ITS2 genetic regions nucleotide sequences by maximum likelihood analysis (Fig. 3). KY and NR formed a closely related group within the clade composed of *B. divergens* and the reindeer *B. divergens*-like organism (Fig. 3). The two *B. odocoilei* isolates formed a separate clade from *B. divergens* and the *B. divergens*-like isolates, which was supported by high bootstrap values (100) in all three analyses (Fig. 3). The rRNA ITS1 genetic region tree further divided the monophyletic group containing *B. divergens* and the *B. divergens*-like isolates into three terminal clades, BdivP, GBRD, and KY (combined with NR), with low bootstrap support (53) (Fig. 3A). The trees inferred from the SSU rRNA ITS2 genetic region and the full rRNA ITS1-5.8S-ITS2 genetic regions sequences showed similar topology (Figs. 3B,C), branching BdivP and GBRD independently, but in a single cluster within the clade containing *B. divergens* and the *B. divergens*-like isolates. A high bootstrap value (92) supported the separation of the group holding NR and KY in the rRNA ITS1-5.8S-ITS2 genetic region tree (Fig. 3C).



A.



B.



C.

Figure 3. Phylogentic analysis of the rRNA ITS1-5.8S-ITS2 genetic region. Maximum likelihood analyses using rRNA ITS1 (**A**), rRNA ITS2 (**B**), and the entire rRNA ITS1-5.8S-ITS2 gene region (**C**) sequence data from three clones each of *Babesia divergens* (BdivP); *Babesia divergens*-like isolates from Kentucky human (KY), Nantucket Island rabbit (NR), and Great Britain reindeer (GBRD); *Babesia odocoilei* from Minnesota caribou (MnBo) and Wisconsin reindeer (WIBo), with *Babesia microti* strains GI (GIBm) and Peabody (PeaBm) as the outgroup.

In Vitro Culture Comparisons

Parasite proliferation

The NR parasite was successfully resuscitated through in vitro culture into both human and cottontail rabbit erythrocytes. *Babesia divergens* was successfully resuscitated through in vitro culture into both human and bovine erythrocytes. Parasites were visible in Giemsa-stained erythrocyte smears of the re-initiated cultures within 6 to 16 days (Table 6). Due to variable multiplication of parasites upon recovery from cryostorage, the number of passages required to achieve the normal subculture schedule varied from 3 to 6 passages among the different cultures (Table 6).

The parasite growth progression, during the 30-day comparative culture period, for each erythrocyte condition, is shown in Fig 4. In most cases, the PPE values peaked on the days subcultures were performed (Fig. 4).

Attempts to subculture NR and *B. divergens* into bovine and cottontail rabbit cells respectively, were unsuccessful. During the three preliminary passages to exchange donor erythroctyes, parasites appeared as dense, dark staining organisms with no visible organelles (Fig. 5 I-J). Hyper-vacuolar parasites were also seen during this period (Fig. 5 S-T). Following the three preliminary passages, parasites were no longer detected.

Babesia divergens in bovine erythrocytes produced the highest PPE values during the experimental period. At subculture parasitemias varied from 4.6 ±1.02 to 10.7 ±1.77, with an average PPE of 5.57 ± 2.53 during the 30 day trial (Fig. 6A). The parasitemia of *B. divergens* in human erythrocytes dropped from an initial high PPE of 8.1 ±0.65 to 9.0

Table 6. Table of resuscitation procedures. List of passage number at time of resuscitation (Thaw psg) and intervals at which the first passage was completed, successive passages were completed, and the number of days post culture re-initiation that the comparative PPE values were taken for NR in human and cottontail rabbit erythrocytes and *B. divergens* in human and bovine erythrocytes.

Parasite	Donor Erythrocyte Host											
	Human				Cottontail Rabbit				Bovine			
	Thaw psg	Days to first Passage	Passage Intervals	Days to Begin	Thaw psg	Days to first Passage	Passage Intervals	Days to Begin	Thaw psg	Days to first Passage	Passage Intervals	Days to Begin
NR774	P23	17	6 days	23	P14	21	5-7 days	33	N/A	N/A	N/A	N/A
B. divergens	P2	19	5 days	24	N/A	N/A	N/A	N/A	P2	8	3-7 days	27

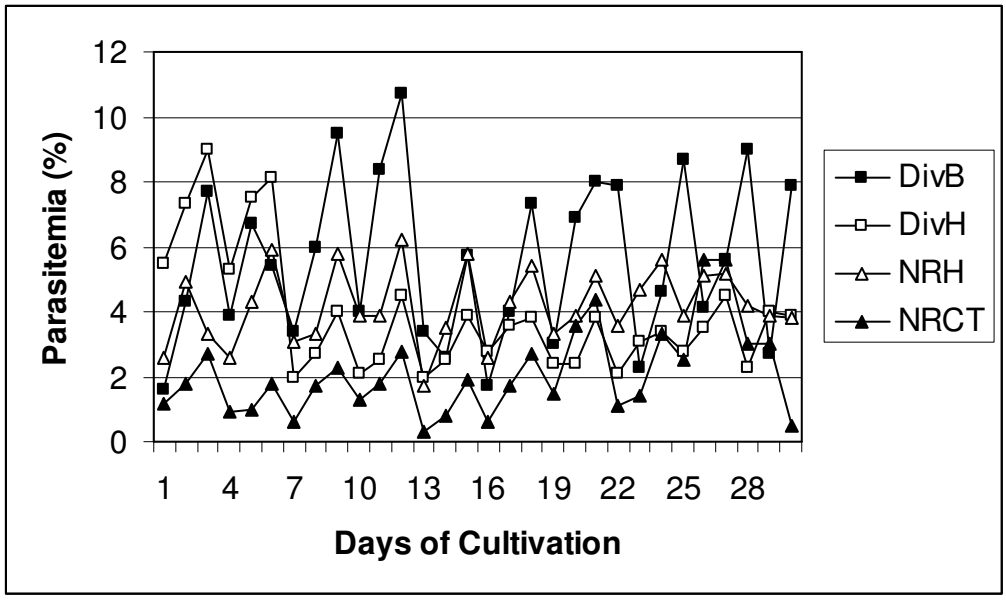


Figure 4. Comparisons of daily percent parasitized erythrocytes. Comparison of culture conditions containing *B. divergens* in bovine erythrocytes (DivB), *B. divergens* in human erythrocytes (DivH), NR in human erythrocytes (NRH) and NR in cottontail rabbit erythrocytes (NRCT) throughout the 30-day experimental period.

±1.04 but maintained a PPE ranging from 3.4 ±0.78 to 4.5 ±0.51 at subculture through the remainder of the study, for an average of 3.91 ±1.89 throughout (Fig. 6A).

NR maintained a PPE in human erythrocytes ranging from 3.3 ±1.36 to 6.2 ±1.41 at subculture throughout, with an average PPE of 4.18 ±1.14 (Fig. 6A). PPE values, at subculture, for NR in eastern cottontail rabbit erythrocytes ranged from 0.5 ±0.41 to 5.6 ±0.86 with an average PPE of 2.12 ±1.28, with an abrupt decline in PPE after the ninth passage (Fig. 6A).

Babesia divergens cultured in bovine erythrocytes proliferated significantly better throughout the 30 day experimental period than *B. divergens* in human erythrocytes, NR in human erythrocytes, and NR in cottontail rabbit erythrocytes ($p \leq 0.05$). *Babesia divergens* cultured in human erythrocytes proliferated significantly better than NR in cottontail erythrocytes ($p \leq 0.05$). The NR parasite cultured in human erythrocytes proliferated significantly better than NR in cottontail erythrocytes ($p \leq 0.05$). The proliferation of *B. divergens* in human erythrocytes was not significantly different from that of NR in human erythrocytes ($p \geq 0.05$).

Media containing fetal bovine serum (HL-F) supported steady growth of both *B. divergens* in bovine erythrocytes and NR in human erythrocytes for the duration of the 30 day trial. At subculture, PPE values of *B. divergens* ranged from 0.6 ±1.14 to 13.43 ±6.15 with an average PPE of 4.81 ±3.13 throughout (Fig. 6B). Percent parasitemias of NR ranged from 1.55 ±0.76 to 3.10 ±0.70 at subculture throughout, with an average PPE of 1.96 ±0.72 (Fig. 6B). *Babesia divergens* cultured in bovine erythrocytes with HL-F

medium proliferated significantly better than NR in human erythrocytes with HL-F medium ($p \leq 0.05$).

Parasite morphology and measurements

Morphological variation was visualized between NR and *B. divergens* through Giemsa-stained erythrocyte smear examination. *Babesia divergens* was seen in single, paired, and accolé forms in bovine erythrocytes, typical of *B. divergens* morphology (Fig. 5 A-D). In addition to the single and paired forms, multiple *B. divergens* organisms were found to exist in single human erythrocytes, with up to 4 parasites present a cell (Fig. 5 E-H). NR was seen in single and paired forms in human and cottontail rabbit erythrocytes (Fig. 5 K,O). A morphologically distinct multiple form of NR was also seen in both host erythrocytes. The multiply infected human or eastern cottontail rabbit erythrocytes contained from 4 to greater than 8 parasites. In many cases, the high number of organisms inhabiting one erythrocyte inhibited parasite quantification and increased the diameter of the infected cell. The average dimensions of each parasite in the different host erythrocytes are included in Table 7. KY and NR were significantly larger in length and width than *B. divergens* in all host erythrocytes ($p \leq 0.05$). The difference in length and width of *B. divergens* in bovine and human erythrocytes was also significant ($p \leq 0.05$). No significant size difference was found between NR in cottontail rabbit erythrocytes, NR in human rabbit erythrocytes, and KY in human erythrocytes ($p \geq 0.05$).

Table 7. List of cultured parasites. The results of cultivation attempts in the various erythrocyte conditions, and dimensions of cultured parasites in the different host erythroctyes. The dimensions of the KY agent measured from a blood smear are also included.

Parasite	Host RBC	Growth Result	Size (µm) Length	Width
B. divergens	Human	+	3.17 ±0.53	1.03 ±0.31
	Bovine	+	2.17 ±0.37	0.75 ±0.25
	Cottontail Rabbit	-	N/A	
NR	Human	+	4.33 ±0.56	1.96 ±0.31
	Bovine	-	N/A	
	Cottontail Rabbit	+	4.16 ±0.48	1.97 ±0.33
KY	N/A	N/A	4.09 ±0.58	1.90 ±0.29

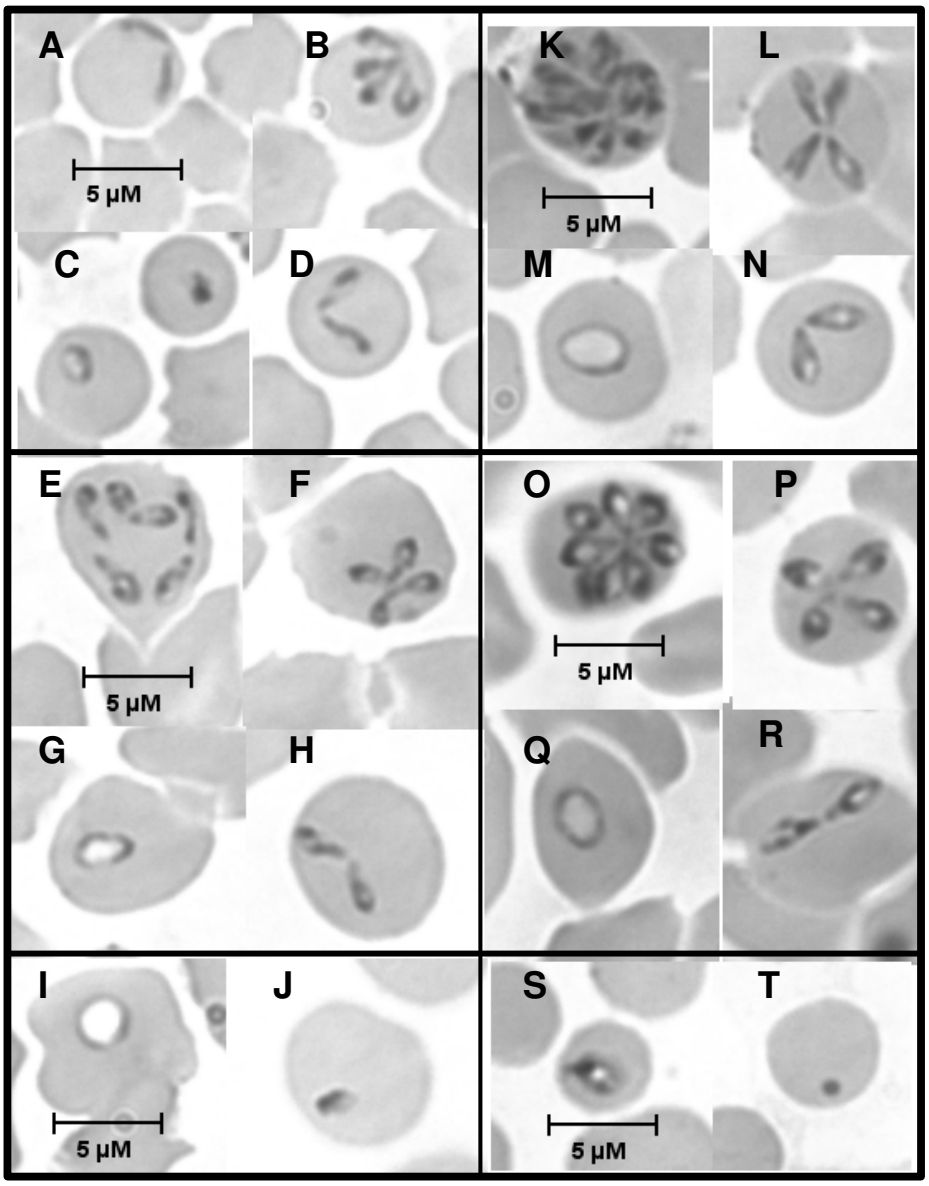
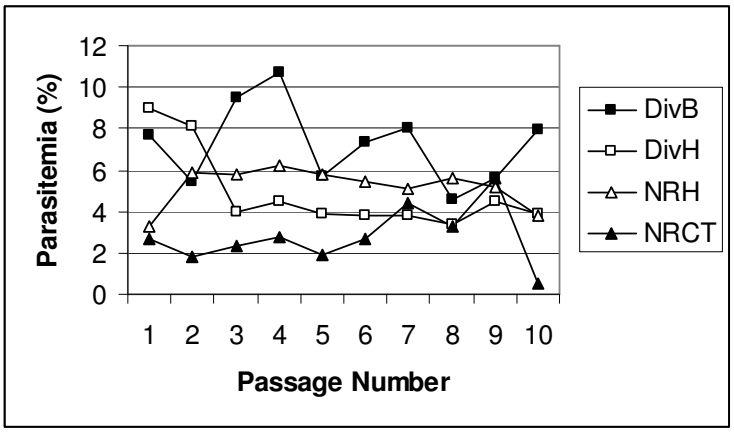
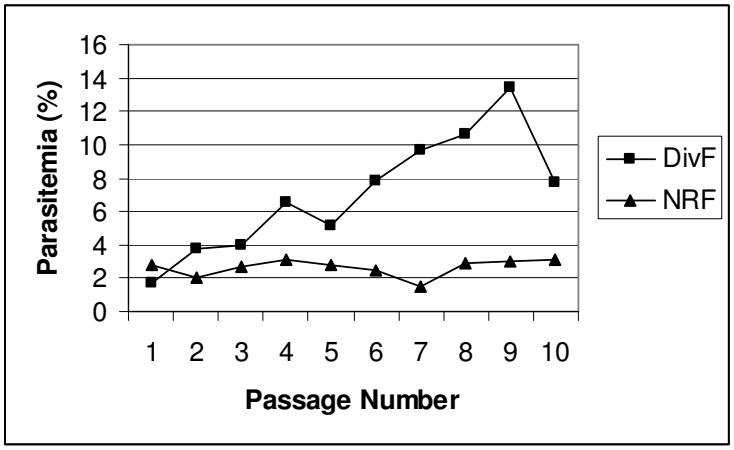


Figure 5. Giemsa-stained smears of erythrocyte culture. **A-D** Paired accolé, multiple, single and paired form of *B. divergens* in bovine erythrocytes. **E-H** Multiple, tetrad, single and paired form of *B. divergens* in human erythrocytes. **I-J** Hyper-vacuolar and dense forms of *B. divergens* in cottontail rabbit erythrocytes. **K-N** Multiple, tetrad, single and paired forms of NR in cottontail erythrocytes. **O-R** Floret, tetrad, single and paired forms of NR in human erythrocytes. **S-T** Hyper-vacuolar and dense forms of NR in bovine erythrocytes.



A.



B.

Figure 6. Comparisons of percent parasitized erythrocytes (PPE)at subculture. **A.** Comparison of PPE values of *B. divergens* in bovine (DivB) and human (DivH) erythrocytes and NR in cottontail rabbit (NRCT) and human (NRH) erythrocytes at each of 10 subcultures. **B.** Comparison of PPE values of *B. divergens* in bovine erythrocytes with fetal bovine supplemented medium (DivF) and NR in human erythrocytes with fetal bovine supplemented medium (NRF).

DISCUSSION AND SUMMARY

During the past few years, cases of human babesiosis occurred in North America with the causative agents distinct from known endemic species, but were similar to the exotic bovine parasite *B. divergens*. Genetic, antigenic, and morphologic evidence relates these recently recognized human agents to this parasite of cattle in Europe (Herwaldt et al. 1996; Beattie et al. 2002; Herwaldt et al. 2004). The first case was fatal and occurred in an elderly asplenic Missouri man (Herwaldt et al. 1996). The remaining two cases, one from Kentucky and the other from Washington State, also occurred in splenectomized men (Beattie et al. 2002; Herwaldt et al. 2004). The agents from the Missouri and Kentucky cases were found to be morphologically similar and genetically identical to each other and an additional North American *Babesia* sp., endemic in eastern cottontail rabbits on Nantucket Island, Massachusetts, (Goethert and Telford 2003a; Holman et al. 2005). The possible existence of *B. divergens* in North America presents a health risk to humans and a potential economic risk to the cattle industry (Cable et al. 2003; Filbin et al. 2003).

Small subunit ribosomal RNA (SSU rRNA) gene sequences are identical between the Kentucky (KY) and Missouri (MO1) isolates (GenBank Accession numbers AY887131 and AY048113, respectively). The piroplasm present in eastern cottontail rabbits is also identical to these human isolates by SSU rRNA gene analysis (Goethert and Telford 2003a). The SSU rRNA gene sequence of these three U.S. organisms is nearly identical to that of *B. divergens* (Purnell isolate, cattle origin) from Great Britain, which differs at only three nucleotide positions (Goethert and Telford 2003a). Interestingly, the

Kentucky patient reported that he had hunted cottontail rabbits prior to becoming ill (Beattie et al. 2002).

Previous evaluations of SSU rRNA gene sequences from *B. divergens* and other *Babesia* spp. provide important considerations when assessing the significance of the three nucleotide differences between *B. divergens* and the U.S. *B. divergens*-like isolate genes. Only four nucleotide positions differ between the SSU rRNA genes of the Great Britain reindeer *B. divergens*-like isolate and Purnell *B. divergens* (GenBank accession numbers AY098643 and U16370, respectively) (Langton et al. 2003). This degree of variation was consistent with the SSU rRNA gene sequence variation found among other cattle *B. divergens* isolates (GenBank accession numbers UO7885 and Z48751), therefore it was concluded that the parasite found in reindeer was *B. divergens* (Langton et al. 2003). However, recent re-analysis of the SSU rRNA genes from these same cattle *B. divergens* isolates found them to be identical to the originally obtained sequence of Purnell *B. divergens* (U16730) (Herwaldt et al. 2003; GenBank accession number AY046576; formerly UO7885 and Z48751). These results suggest that no variation exists in the SSU rRNA gene within this species, which would then suggest that the four nucleotide differences found between *B. divergens* and the reindeer isolate SSU rRNA genes may, in fact, signify delineation of species. This would further suggest that the three differences in SSU rRNA gene sequences of *B. divergens* and the U.S. *B. divergens*-like isolates, and the seven differences between the Great Britain reindeer *B. divergens*-like and the U. S. *B. divergens*-like isolates (GenBank accession numbers U16370, AY048113, and AY098643, respectively) also signify delineation of species.

Thus, the SSU rRNA data suggests separation of *B. divergens* and the U.S. isolate. Biologically, the mammalian hosts of *B. divergens* are well documented and include cattle, man, and gerbils (Homer et al. 2000), but rabbits have not been documented. The U.S. parasite infects rabbits and man, but not cattle (Herwaldt et al. 1996; Beattie et al. 2002; Goethert and Telford 2003a). The mammalian host disparity between the two organisms and the SSU rRNA data indicate that these two parasites are not conspecific.

The rRNA ITS1 and ITS2 genetic regions are less conserved than the SSU rRNA genes due to reduced structure-function constraints placed upon these non-coding genes. Therefore, ITS data have been used to delineate species within a genus (Zahler et al. 1998; Berzunza-Cruz et al. 2002). The consensus sequences of the rRNA ITS1 and ITS2 regions of the Nantucket Island rabbit and Kentucky human *B. divergens*-like isolates were identical in size and nucleotide sequence, supporting conspecificity of these two organisms (Tables 3,4). Both *B. divergens* and the reindeer *B. divergens*-like isolate possessed ITS sequences variant to each other and to that of the U.S. isolate (Table 4). In both ITS regions, the Great Britain reindeer *B. divergens*-like was more similar to the geographically distant U.S. human *B. divergens*-like isolates than to the Great Britain *B. divergens* (Table 4). The *B. microti* isolates (Peabody and GI) included in this study have ITS1 and ITS2 regions of the same size and nearly identical sequence (98.9% and 99.5 %, respectively) (Table 4). In contrast, the *B. odocoilei* isolates show more rRNA ITS sequence variation between each other than is seen between the U.S. *B. divergens*-like isolates and *B. divergens* and the reindeer *B. divergens*-like isolate from Great Britain (Table 4).

The usefulness of the rRNA ITS1 and ITS2 genetic regions as taxonomic genetic markers for members of the family Babesiidae has not been definitively demonstrated for any species except *B. canis.* *Babesia canis* isolates are differentiated into three subspecies based on biologic characteristics including vector specificity, immunogenicity, and pathogenicity (Uilenberg et al. 1989; Hauschild et al. 1995). The rRNA ITS1 and ITS2 genetic region sequence analysis also supports the differentiation of these three subspecies, with percent identities ranging from 70 to 82 between subspecies and 97.9 to 100.0 within subspecies for the full rRNA ITS1-5.8S-ITS2 genetic region (Zahler et al. 1998). In contrast, intragenic comparisons between the *B. canis* subspecies and *B. caballi* show lower percent identities (66-69) in this genomic region (Zahler et al. 1998). Similarly, percent identities ranged from 67-69 in the rRNA ITS1 genetic region and from 70-73 in the rRNA ITS2 genetic region when *B. odocoilei* was compared to *B. divergens* and the *B. divergens*-like isolates. However, percent identities were much higher between *B. divergens* and the *B. divergens*-like isolates (89-94) (Table 4). The greater rRNA ITS genetic region sequence variation seen within isolates of *B. odocoilei,* compared to the lower values found between *B. divergens* and the U.S. isolates (KY and NR), would support an argument for conspecificity of the latter two organisms, but conflicts with the SSU rRNA data and biologic evidence, which distinguish these organisms.

Considered together, all the data from *Babesia* spp. suggest that the amount of rRNA ITS genetic region variation within a given species may differ depending on the species. In all cases, intragenic comparisons of rRNA ITS genetic regions showed more variation than was found within an isolate. However, as discussed above, the amount of SSU

rRNA ITS genetic region variation found within an isolate was not consistent. In some isolates, the SSU rRNA ITS genetic regions were the same size and showed only slight nucleotide variation, as seen among the clones of the Kentucky human and Nantucket Island rabbit isolates and those of *B. divergens*. This high degree of similarity was also seen among the *B. microti* isolates, where three rRNA ITS1-5.8S-ITS2 genetic region clones of the *B. microti* GI strain were identical, and were also nearly identical to that of the *B. microti* Peabody strain. On the other hand, differences in both size and sequence of the rRNA ITS genetic regions were found between the two *B. odocoilei* isolates and, moreover, within the three *B. odocoilei* and the three reindeer *B. divergens*-like parasite clones. The likely existence of multiple SSU rRNA coding regions, as determined for *B. bigemina* and *B. bovis*, may explain the sequence variation found among the clones of the isolates compared in this study (Dalrymple 1990; Reddy et al. 1991).

Although the 5.8S rRNA gene is considered too small in length to be a reliable candidate for phylogenetic analysis, it is noteworthy that *B. divergens* and the *B. divergens*-like isolates possess identical 5.8S gene sequences. These results are consistent with those of *Cyclospora cayetanensis* and *Cyclospora papionis*, which also share identical 5.8S rRNA gene sequences, but differ in the rRNA ITS1 genetic region (Olivier et al. 2001). In fact, even parasites taxonomically assigned to distinct genera may share identical 5.8S rRNA gene sequences, as seen in *Hammondia heydorni*, *Hammondia hammondi*, *Neospora caninum,* and *Toxoplasma gondii* (Blast search; GenBank accession numbers AF096501, AF508030, AF432123, and L49390, respectively). The two *B. microti* strains also shared identical 5.8S rRNA gene sequences. Interestingly, among the *B. canis* subspecies, variation in the 5.8S rRNA gene is observed. The 5.8S rRNA gene sequence ranged from

96.9-97.5 percent identity among the three subspecies (GenBank accession numbers AF394535, AF394534, AF394533) and 94.3-95.6 between *B. canis* and *B. caballi* (GenBank accession number AF394536). In summary, as discussed for the rRNA ITS regions, the degree of sequence variation in the 5.8S rRNA gene may also depend on the *Babesia* species.

Phylogenetic analyses further support the conspecificity of the Kentucky human and Nantucket Island rabbit *B. divergens*-like isolates, and the close evolutionary relationships among this parasite, *B. divergens* and the reindeer *B. divergens*-like parasite. Three maximum likelihood trees constructed from the rRNA ITS1 genetic region, rRNA ITS2 genetic region or the entire rRNA ITS1-5.8S-ITS2 genetic region sequences, indicate conspecificity of the U.S. rabbit and human parasites, with each placing these isolates together into distinct terminal clades. However, in all three trees, a similar topology combines the *B. divergens* and *B. divergens*-like clones into one non-terminal monophyletic clade, which is separated from *B. odocoilei*.

The usefulness of the rRNA ITS genetic regions for distinguishing among species has been established for a number of protozoan genera, including *Sarcocystis, Cyclospora, Cryptosporidium,* and *Leishmania* (Marsh et al. 1999; Miller et al. 2001; Olivier et al. 2001; Morgan-Ryan, et al. 2001; Berzunza-Cruz et al. 2002). However, in the present study, diverse *Babesia* spp. exhibited different degrees of rRNA ITS genetic region sequence variability within an isolate, so that delimitations on rRNA ITS genetic region variability that define a *Babesia* species were not possible. Thus, the global use of rRNA ITS genetic region data in delineating *Babesia* spp. is not likely to be practical if based on

standards derived from intragenic and/or intraspecies variation. The value of rRNA ITS sequence data lies in identifying conspecific *Babesia* isolates, such as with the parasite from the cottontail rabbit and the agent of human babesiosis in this study.

In addition to molecular comparisons, animal inoculations studies were conducted to evaluate the in vivo infectivity of the NR isolate (Holman et al. 2005). Cryopreserved culture inocula of the NR isolate and *B. divergens* were employed to evaluate infectivity of these two organisms for cattle. Cattle are refractory to NR infection. However, control animals inoculated with *B. divergens* became infected as determined by Giemsa stained smear examination, PCR, and in vitro culture (Holman et al. submitted).

The current study shows that similar infectivity determinations can be made through in vitro methods. Thus, differences in host erythrocyte suitability were observed among the parasites cultured in vitro in this study. Despite multiple attempts, the rabbit parasite would not grow in bovine cells that concurrently supported *B. divergens* proliferation, and *B. divergens* would not grow in cottontail rabbit cells that concurrently supported NR proliferation. This result shows a distinct difference in the host erythrocyte specificity between NR and *B. divergens*, and supports the in vivo findings.

In this study, *B. divergens* cultured in bovine erythrocytes proliferated at a higher level of parasitemia than in human erythrocytes or compared to NR in human or cottontail erythrocytes. The importance of quality and origin of both erythrocytes and sera used for *Babesia* spp. in vitro culture, is well documented and is evident in this experiment (Sunuga et al. 2002; Zintl et al. 2004; Holman et al. 2005). The bovine erythrocytes and serum available for *B. divergens* culture were from a donor animal on site, which was

previously selected based on the superior performance of its erythrocytes and serum to support *B. divergens* growth in vitro. Bovine erythrocytes and serum, therefore, were expected to provide optimal support for parasite growth in vitro.

In contrast, the human erythrocytes were from a commercial source and likely were obtained from different donors from lot to lot. The human serum was acquired as a single lot batch and therefore constant throughout the in vitro studies. In addition, this lot was previously shown to support NR in vitro (Holman et al. 2005). Nevertheless, the human erythrocytes and serum were not autologous as were the bovine components. Furthermore, it was observed during these studies that parasite growth declined in human erythrocytes used four weeks after blood collection. It was previously noted that cultures of *B. gibsoni* must be replenished with fresh canine erythrocytes (Zweygarth and Lopez-Rebollar 2000; Sunuga et al. 2002). Since human blood must be safety tested prior to sale and release, prompt replacement of donor human erythrocyte stocks was occasionally problematic. In fact, the decline in PPE values seen at the third passage of *B. divergens* grown in human erythrocytes coincided with use of a new shipment of human blood and might, therefore, be due to erythrocyte quality. As mentioned above, the culture suitability of each lot is likely to be variable since the donors varied. The importance of using erythrocytes from a suitable donor in cultures of *Babesia* species is well documented (Canning and Winger 1987; Holman et al. 1998).

Similarly, the generally lower PPE values seen in NR in cottontail rabbit erythrocyte cultures may be attributed to both erythrocyte quality and serum compatibility (Fig. 6a). The supply of cottontail rabbit erythrocytes was limited to blood drawn from wild-caught

rabbits. During this study, it was observed that cottontail rabbit erythrocytes did not maintain integrity under storage as well as human and bovine erythrocytes. Supplementation of culture medium with cottontail rabbit serum was not only impractical, but not feasible as well. The use of human serum in lieu of cottontail rabbit may have contributed to poor condition of the rabbit erythrocytes in culture, thereby affecting parasite proliferation.

Fetal bovine serum supplementation of culture medium for bovine *Babesia* spp. has been avoided due to inhibitory effects on parasite growth presumably associated with the phenomenon of inverse age resistance in bovine babesiosis (Levy et al. 1982). Recent reports, however, show that these effects may not be seen in cultures of *B. divergens*, which may be grown in the presence of fetal bovine serum (Ben Musa and Phillips 1991; Chauvin et al. 2002; Malandrin et al. 2004). Current experimentation focusing on the role of the calf spleen in reverse age resistance has reduced the conceived importance of fetal bovine serum in this phenomenon in regards to *B. divergens* (Zintl et al. 2004; Zintl et al. 2005). Previously, primary cultures of NR were not successfully initiated in the presence of fetal bovine serum (Holman et al., 2005). In the current study, established cultures of both *B. divergens* and NR774 could be successfully subcultured and maintained using fetal bovine serum supplemented medium (Fig. 6b). This difference may be explained by different culture requirements between primary and established *Babesia* species cultures. For example, fetal bovine serum is necessary for the initiation of *B. equi* cultures, but after establishment, the parasite proliferates in medium containing normal adult horse serum (Holman et al. 1994b). Similarly, Zweygarth and Lopez-Rebollar (2000) found that hypoxanthine was required for initiation of *B. equi* cultures,

but hypoxanthine could be satisfactorily replaced by adenosine or guanosine in established cultures.

A major distinguishing characteristic among *Babesia* species is the size of the piroplasm. The parasites are grouped into large and small babesia depending on whether an individual piroplasm is larger or smaller than 2.5 µm, respectively, in length during the paired merozoite stage. This study further defines NR and *B. divergens* as separate species based on size differences. *Babesia divergens* is classified as a small *Babesia* species, with merozoites measuring from 1.0 up to 2.5 µm in length in bovine blood (Levine 1985). When cultured in bovine erythrocytes, *B. divergens* parasites were consistent in size to that reported in vivo. However, *B. divergens* cultured in human erythrocytes exceeded the small *Babesia* species size limitations, being significantly larger with an average size of 3.17 ($p \leq 0.05$) (Table 7). In contrast, the size of NR parasites remained comparable whether cultured in human or rabbit erythrocytes, with average lengths of 4.33 µm and 4.16 µm, respectively (Table 7). Regardless of host erythrocyte, NR parasites were significantly larger than *B. divergens* ($p \leq 0.05$) and consistent in size with the KY parasite (4.09 µm) ($p \geq 0.05$) (Table 7).

The observed difference in size of *B. divergens* parasites may be attributed to differences in host erythrocyte size. Previously, *B. divergens* parasites in human blood were described as larger, up to 3 µm, than *B. divergens* in bovine blood, up to 2.5 µm (Gorenflot et al. 1998; Levine 1985). Human and cottontail rabbit erythrocytes are approximately 9 µm in diameter, which correlates with the consistent size of NR when cultured in either human or rabbit erythrocytes. Bovine erythrocytes are considerably

smaller at approximately 6 µm in diameter. In sum, when NR and *B. divergens* are measured in the same host erythrocytes, the size discrepancy between them is significant ($p \leq 0.05$), indicating that these organisms are distinct.

Other morphological differences exist between *B. divergens* and the NR774 parasite (Fig 5). *Babesia divergens* was often observed in its typical accolé position in bovine erythrocytes, but not in human erythrocytes, which agrees with previous observations of *B. divergens* morphology in human erythrocytes (Gorenflot et al. 1991; Pudney 1984). Transmission electron microscopy shows that *B. divergens* parasites do not protrude against the membrane of human erythrocytes, but instead occupy a subcentral position (Gorenflot et al. 1991). In the current study, Maltese cross or tetrad forms within either erythrocyte type were rare, but were more common in human erythrocytes.

In both human and cottontail rabbit erythrocytes, NR parasites were also found as single, paired, and tetrad forms, with paired merozoites occasionally in the accolé positions, as noted for *B. divergens* in bovine erythrocytes(Fig 5 K-R). Additionally, in excess of four NR parasites were often found within a single erythrocyte, often exceeding eight parasites per cell and sometimes appearing in an unusual floret-like formation (Fig. 5 K,O). A similar formation is reported as a "uniform array" in *B. odocoilei* parasites cultured in caribou erythrocytes (Holman et al. 1994a). *Babesia divergens* was not observed in this form in either bovine or human erythrocytes.

According to the rRNA ITS1 and ITS2 genetic region sequence data collected in this study, the KY and NR agents are conspecific. The rRNA ITS1 and ITS2 genetic region data are less clear in defining the relationship between *B. divergens* and the North

American *Babesia* sp. However, the differences in host erythrocyte utility between NR and *B. divergens* and the morphological variation found between them when cultured in human erythrocytes distinguish these organisms as distinct species. These results clearly demonstrate the value of in vitro methodology in characterizing *Babesia* spp. and provide a more practical and humane method than in vivo experimentation. Furthermore, the rRNA ITS1-5.8S-ITS2 sequence data and differential host erythrocyte specificity data from this study show that the NR agent is distinct from *B. divergens*.

REFERENCES

Adam RD, Ortega YR, Gilman RH, Sterling CR (2000) Intervening transcribed spacer region 1 variability in *Cyclospora cayatenensis*. J Clin Microbiol 38:2339-2343

Beattie JF, Michelson ML, Holman PJ (2002) Acute babesiosis caused by *Babesia divergens* in a resident of Kentucky. N Engl J Med 347:697-698

Benezra D, Brown AE, Polsky B, Gold JW, Armstrong D (1987) Babesiosis and infection with human immunodeficiency virus (HIV). Ann Intern Med 107: 944

Ben Musa N, Phillips RS (1991) The adaptation of three isolates of *Babesia divergens* to continuous culture in rat erythrocytes. Parasitology 103:165-170

Berzunza-Cruz M, Cabrera N, Crippa-Rossi M, Cabrera TS, Perez-Montfort R, Becker I (2002) Polymorphism analysis of the internal transcribed spacer and small subunit of ribosomal RNA genes of *Leishmania mexicana*. Parasitol Res 88:918-925

Cable RG, Leiby DA (2003) Risk and prevention of transfusion-transmitted babesiosis and other tick borne diseases. Curr Opinion in Hematol 10:405-411

Cacciò S, La Rosa G, Pozio E (1997) The ß-tubulin gene of *Cryptosporidium parvum*. Mol Biochem Parasitol 89:307-311

Canning EU, Winger CM *In* AER Taylor, Baker JR (eds.), In vitro methods for parasite cultivation, (1987) Academic Press, London, England.

Chauvin A, Valentin A, Malandrin L, L'Hostis M (2002) Sheep as a new experimental host for *Babesia divergens*. Vet Res 33:429-433

Collins NE, Allsopp BA (1999) *Theileria parva* ribosomal internal transcribed spacer sequences exhibit extensive polymorphism and mosaic evolution: application to the characterization of parasites from cattle and buffalo. Parasitology 118:541-551

Dalrymple BP (1990) Cloning and characterization of the rRNA genes and flanking regions from *Babesia bovis*: use of the genes as strain discriminating probes. Mol Biochem Parasitol 43:117-124

Droleskey RE, Holman PJ, Waldrup KA, Corrier DE, Wagner GG (1993) Ultrastructural characteristics of *Babesia odocoilei* in vitro. J Parasitol 79:424-434

Duh D, Petrovec M, Avsic-Zupanc T (2001) Diversity of *Babesia* infecting European sheep ticks (*Ixodes ricinus*). J Clin Microbiol 39:3395-3397

Emerson R, Held AA (1969) *Aquilinerella fermentans* gen. et sp. N., a phycomycete adapted to stagnant waters. Am J Bot 56:1103

Filbin MR, Mylonakis EE, Callegari L, Legome E (2003) Babesiosis. J Emerg Med. 20: 21-24

Goethert HK, Telford SR III (2003a) Enzootic transmission of *Babesia divergens* among cottontail rabbits on Nantucket Island, Massachusetts. Am J Trop Med Hyg 69:455-460

Goethert HK, Telford SR III (2003b) What is *Babesia microti*? Parasitology 127:301-309

Goff WL, Jessup DA, Waldrup KA, Thomford JW, Conrad PA, Boyce WA, Gorham JR, Wagner GG (1993) The isolation and partial characterization of a *Babesia* sp. from desert bighorn sheep (*Ovis canadensis nelsoni*) J Euk Microbiol 40:237-243

Gorenflot A, Brassuer P, Precigout E, L' Hostis M, Marchand A, Schrevel J (1991) Cytological and immunological responses to *Babesia divergens* in different hosts: ox, gerbil, man. Parasitol Res 77:3-12

Gorenflot A, Moubri K, Precigout E, Carcy B (1998) Human babesiosis. Ann Trop Med Parasitol 92:489-501

Grey J, von Stedingk LV, Gürtelschmid M, Granström M (2002) Transmission studies of *Babesia microti* in *Ixodes ricinus* ticks and gerbils. J Clin Microbiol 40:1259-1263

Hauschild S, Shayan P, Schein E (1995) Characterization and comparison of merozoite antigens of different *Babesia canis* isolates by serological and immunological investigations. Parasitol Res 81: 638-642

Healy GR (1976) *Babesia* infections in man. Hosp Pract 14:107-116

Herwaldt BL, Persing DH, Precigout EA, Goff WL, Mathiasen DA, Taylor PW, Eberhard ML, Gorenflot AR (1996) A fatal case of babesiosis in Missouri: identification of another piroplasm that infects humans. Ann Int Med 124:643-650

Herwaldt BL, Cacciò S, Gherlinzoni F, Aspock H, Slemenda SB, Piccaluga P, Matinelli G, Edelhofer R, Hollenstein U, Poletti G, Pampiglione S, Loschenberger K, Tura S, Pieniazek NJ (2003) Molecular characterization of a non-*Babesia divergens* organism causing zoonotic babesiosis in Europe. Emerg Infect Dis 9:942-948

Herwaldt BL, de Bruyn G, Pienazek NJ, Homer M, Lofy KH, Slemenda SB, Fritsche TR, Persing DH, Limaye AP (2004) *Babesia divergens*-like infection, Washington State. Emerg Infect Dis 10:622-629

Holman PJ, Frerichs WM, Chieves L, Wagner GG (1993a) Culture confirmation of the carrier status of *Babesia caballi*-infected horses. J Clin Microbiol 31:698-701

Holman PJ, Waldrup KA, Droleskey RE, Corrier DE, Wagner GG (1993b) In vitro growth of *Babesia bovis* in white-tailed deer (*Odocoileus virginianus*). J Parasitol 79:233-237

Holman PJ, Petrini KR, Rhyan JC, Wagner GG (1994a) Culture isolation and partial characterization of a *Babesia* from a North American woodland caribou (*Tarandus rangifer caribou*). J Wildl Dis 30:195-200

Holman PJ, Chieves L, Frerichs WM, Olsen D, Wagner GG (1994b) *Babesia equi* erythrocytic stage continuously cultured in an enriched medium. J Parasitol 80:232-236

Holman PJ, Becu T, Bakos E, Polledo G, Cruz D, Wagner GG (1998) *Babesia equi* field isolates cultured from horse blood using a microcentrifuge method. J Parasitol 84:696-699

Holman PJ, Madeley J, Craig TM, Allsopp BA, Allsopp MTEB, Petrini KR, Waghela SD, Wagner GG (2000) Antigenic, phenotypic and molecular characterization confirms *Babesia odocoilei* isolated from three cervids. J Wildl Dis 36:518-530

Holman PJ, Bendele KG, Schoelkopf L, Jones-Witthuhn RL, Jones SO (2003) Ribosomal RNA analysis of *Babesia odocoilei* isolates from farmed reindeer (*Rangifer tarandus tarandus*) and elk (*Cervus elephus canadensis*) in Wisconsin. Parasitol Res 91:378-383

Holman PJ, Spencer AM, Drolesky RE, Goethert H, Telford S (2005) In vitro cultivation of a zoonotic *Babesia* sp. isolated from eastern cottontail rabbits (*Sylvilagus floridanus*) on Nantucket Island, Massachusetts. J Clin Microbiol

Holman PJ, Spencer AM, Telford SR, Allen A, Goff WL (submitted) Partial characterization of a zoonotic Babesia divergens-like parasite from the northeastern United States: results of an in vivo study in cattle. Am J Trop Med Hyg

Homer MJ, Auguilar-Delfin I, Telford SR, Krause PJ, Persing DH (2000) Babesiosis. Clin Microbiol Rev 13:451-469

Kjemtrup AM, Conrad PA (2000) Human babesiosis: an emerging tick-borne disease. Int J Parasitol 30:1323-1337

Langton C, Gray JS, Waters PF, Holman PJ (2003) Naturally acquired babesiosis in a reindeer (*Rangifer tarandus tarandus*) herd in Great Britain. Parasitol Res 89:194-198

Levine ND (1973) Protozoan parasites of domestic animals and of man. Burgess Publishing Company. Minneapolis, Minnesota pp 317-343

Levine ND (1985) Veterinary protozoology. The Iowa State University Press. Ames, Iowa pp 291-328

Levy MG, Ristic M (1980) *Babesia bovis*: continuous cultivation in a microaerophilous stationary phase culture. Science 207:1218-1220

Levy MG, Clabaugh G, Ristic (1982) Age resistance in bovine babesiosis: role of blood factors in resistance to *Babesia bovis*. Infect Imm 37:1127-1131

Lux JZ, Weiss D, Lindon JV, Kessler D, Herwaldt BL, Wong SJ, Keithly J, Della-Latta P, Scully BE (2003) Transfusion-associated babesiosis after heart transplant. Emerg Infect Dis 9:116-119

Malandrin L, L'Hostis M, Chauvin A (2004) Isolation of *Babesia divergens* from carrier cattle blood using in vitro culture. Vet Res 35:131-139

Marsh AE, Barr BC, Tell L Bownman DD, Conrad PA, Ketcherside C, Freen T (1999) Comparison of the internal transcribed spacer, ITS-1, from *Sarcocystis falcatula* isolates and *Sarcocytis neurona* J Parasitol 85: 750-757

Mehlhorn H, Schein E (1984) The piroplasms: life cycle and sexual stages. Adv Parasitol 23:37-103

Miller MA, Crosbie PR, Sverlow K, Hanni K, Barr BC, Kock N, Murray MJ, Lowenstine LJ, Conrad PA (2001) Isolation and characterization of *Sarcocystis* from brain tissue of a free living southern sea otter (*Enhydra lutri nereis*) with fatal meningoencephalitis. Parasitol Res 87: 252-257

Morgan-Ryan UM, Monis P, Possenti A, Cristanti A, Spano F (2001) Cloning and phylogenetic analysis of the ribosomal internal transcribed spacer-1 (ITS1) of *Cryptosporidium wrairi* and its relationship to *C. parvum* genotypes Parasitologia 43:159-163

Mylonakis MD (2001) When to suspect and how to monitor babesiosis. Am Fam Physician 63:1969-1974

Olivier C, van de Pas P, Lepp PW, Yoder K, Relman DA (2001) Sequence variability in the first transcribed spacer region within and among *Cyclospora* species is consistent with polyparasitism. Int J Parasitol 31: 1475-1487

Pearson WRT, Wood T, Zhang Z, Miller W (1997) Comparison of DNA sequences with protein sequences. Genomics 46:24-36

Peisman J, Spielman P, Etkind P, Ruebush T, Juranek DD (1979) Role of deer in the epizootiology of *Babesia microti* in Massachusetts, USA. J Med Entomol 15:537-540

Peisman J, Karakshian SJ, Lewengrub S, Rukdzinska MA, Spielman A (1986) Development of *Babesia microti* sporozoites in adult *Ixodes dammini*. Int J Parasitol 16:381-385

Persing DH, Herwaldt BL, Glaser C, Lane RS, Thomford JW, Matheisen D, Krause PJ, Phillip DF, Conrad PA (1995) Infection with a *Babesia*-like organism in northern California. N Engl J Med 332:298-303

Piccaluga PP, Poletti G, Martinelli G, Gherlinzoni F (2004) *Babesia* infection in Italy. Lancet 4:212

Prichard R, Tait A (2001) The role of molecular biology in veterinary parasitology. Vet Parasitol 98:169-194

Pudney M (1984) Cultivation of *Babesia divergens* in bovine and human erythrocytes in vitro. British Society for Parasitology Proceedings. Parasitology 89:ixxvi.

Purnell RE, Brocklesby DW, Hendry DJ, Young ER (1976) Separation and recombination of *Babesia divergens* and *Ehrlichia phagocytophila* from a field case of redwater from Erie. Vet Rec 99:415-417

Quick RE, Herwaldt BL, Thomford JW, Garnett ME, Eberhard ML, Wilson MW, Spach DH, Dickerson JW, Telford SR, Steingart KR, Pollock R, Persing DH, Kobayashi

JM, Juranek DD, Conrad PA (1993) Babesiosis in Washington State: a new species of *Babesia*? Ann Int Med 119:284-290.

Reddy GR, Chakrabarti D, Yowell CA, Dame JB (1991) Sequence microheterogeneity of the three small subunit ribosomal RNA genes of *Babesia bigemina*: expression in erythrocyte culture. Nucleic Acids Res 19:3641-3645

Rudzinska MA, Trager W (1976) Formation of merozoites in intraerythrocytic *Babesia microti*: an ultrustructural study. Can J Zool 55: 928-938

Ruebush MJ, Hanson WL (1979) Susceptability of five strains of mice to *Babesia microti* of human origin**.** J Parasitol 65:4630-4633

Saito-Ito A, Yano Y, Dantrakool A, Hashimoto T, Takada N (2004) Survey of rodents and ticks in human babesiosis emergence area in Japan: first detection of *Babesia microti*-like parasites in *Ixodes ovatus*. J Clin Microbiol 42:2268-2270

Sambrook JE, Fritsch F, Maniatis T (1989) Molecular cloning, a laboratory manual, 2nd edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Scholtens RG, Braff EH, Healy GR, Gleason N (1968) A case of babesiosis in man in the United States. Am J Trop Med Hyg 17:810-813

Simpson CF, Bild CE, Stoliker HE (1963) Electron microscopy of canine and equine *Babesia*. Am J Vet Res 24:408-414

Simpson CF, Kirkman WW, Kling JM (1967) Comparative morphologic features of *Babesia caballi* and *Babesia equi*. Am J Vet Res 28:1693-1967

Skrabalo Z, Deanovic Z (1957) Piroplasms in man: report on a case. Doc Med Geogr Trop 9:11-16

Smith T, Kilborne FH (1893) Investigations into the nature, causation and prevention of Texas or southern cattle fever. Bulletin of Bureau of Animal Industry U.S. Dept of Agriculture, Washington, D.C. pp 177-304

Sunuga F, Namikawa K, Kanno Y (2002) Continuous in vitro culture of erythrocytic stages of *Babesia gibsoni* and virulence of the cultivated parasite. J Vet Med Sci 64:571-575

Swofford D L (2002) Paup*. Phylogenetic analysis using parsimony (*and other methods). 4th ed. Sinauer Associates, Sunderland, Massachusetts

Telford SR, Goethert H (2004) Emerging tick-borne infections: rediscovered and better characterized, or truly new? Parasitology 129:887-4236

Trager W (1971) Further studies on the effects of antipantothenates on malaria parasites (*Plasmodium coatneyi* and *P. falciparum*) in vitro. J Protozool 18:232,239

Trager W, Jenson JB (1976) Human malaria parasites in continuous culture. Science 193:673-675

Uilenberg G, Franssen FFJ, Perié NM, Spamjer AAM (1989) Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Vet Q 11:33-40

Varynen R, Tuomi J (1982) Continuous in vitro cultivation of *Babesia divergens*. Acta Vet Scand 23:471-472

Vega CA, Buening GM, Green TJ, Carson CA (1985) In vitro cultivation of *Babesia bigemina*. Am J Vet Res 46:416-420

Waldrup KA, Moritz J, Baggett D, Magyar S, Wagner GG (1992) Monthly incidence of *Theileria cervi* and seroconversion to *Babesia odocoilei* in white-tailed deer (*Odocoiles virginanus*) in Texas. J Wildl Dis 28:457-459

Wall R, Shearer D (2001) Veterinary ectoparasites: biology, pathology and control, 2nd ed. Blackwell Science, Ames, Iowa pp 58-64

Zahler M, Schein E, Rinder H, Gothe R (1998) Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. Parasitol Res 84: 544-548

Zintl A, Skerrett HE, Grey JS, Brophy PO, Mulcahy G (2004) *Babesia divergens* (phylum Apicomplexa) in vitro growth in the presence of calf serum. Vet Parasitol 112:127-130

Zintl A, Grey JS, Skerrett HE, Mulcahy G (2005) Possible mechanisms underlying age-related resistance to bovine *Babesias*. Parasite Immunol 27: 115-120

Zweygarth E, Lopez-Rebollar LM (2000) Continuous in vitro cultivation of *Babesia gibsoni*. Parasitol Res 86:905-907

VITA

Angela M. Spencer
Texas A&M University
Department of Veterinary Pathobiology
College Station, Texas

EDUCATION

2005 M.S. Veterinary Parasitology, Texas A&M University, College Station Texas.
1997 B.A. Psychology, Baylor University, Waco, Texas.

PUBLICATIONS

Holman PJ, Spencer AM, Drolesky RE, Goethert H, Telford S (2005) In vitro cultivation of a zoonotic *Babesia* sp. isolated from eastern cottontail rabbits (*Sylvilagus floridanus*) on Nantucket Island, Massachusetts. J Clin Microbiol

Holman PJ, Spencer AM, Telford SR, Allen A, Goff WL (submitted) Partial characterization of a zoonotic *Babesia divergens*-like parasite from the northeastern United States: results of an in vivo study in cattle. Am J Trop Med Hyg

Spencer AM, Goethert HK, Telford SR, Holman PJ (submitted) In vitro host erythrocyte specificity and differential morphology of *Babesia divergens* and a zoonotic *Babesia* sp. from eastern cottontail rabbits (*Sylvilagus floridanus*). J Parasitol