COMPARATIVE INFECTIVITY OF BABESIA DIVERGENS AND A ZOONOTIC BABESIA DIVERGENS–LIKE PARASITE IN CATTLE

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Abstract. Babesia divergens–like parasites identified in human babesiosis cases in Missouri and Kentucky and in eastern cottontail rabbits (Sylvilagus floridanus) on Nantucket Island, Massachusetts, share identical small subunit ribosomal RNA gene sequences. This sequence is 99.8% identical to that of Babesia divergens, suggesting that the U.S. parasite may be B. divergens, a causative agent of human and bovine babesiosis in Europe. Holstein-Friesian calves were inoculated with cultured Nantucket Island Babesia sp. (NR831) and B. divergens parasites and monitored by clinical signs, Giemsa-stained blood films, PCR, and culture. The NR831 recipients did not exhibit clinical signs of infection and remained negative for all assays. The B. divergens recipients developed clinical infections and became positive by all assays. NR831 recipients were fully susceptible upon challenge inoculation with B. divergens. This study confirms that the Nantucket Island Babesia sp. is not conspecific with B. divergens based on host specificity for cattle.

INTRODUCTION

Members of the Babesiidae are protozoan parasites in the order Piroplasmorida and subphylum Apicomplexa. The agents of babesiosis, these tick-transmitted blood parasites are found in mammalian hosts throughout the world. Some species are host-specific, and others are more promiscuous and may even infect hosts across taxonomic classes including humans. Thus, babesiosis, the disease associated with these parasites, is of economic, veterinary, and medical importance.

Human babesiosis is an emerging tick-borne disease.1,2 Most human cases in the United States are caused by Babesia microti,3 primarily a parasite of rodents, whereas cases in Europe are usually caused by Babesia divergens,4 primarily a parasite of cattle but also experimentally in gerbils.5 However, over the past several years, additional human infections in the United States have been reported, and molecular characterization confirms that they are not B. microti.6–11 Of these, three cases reported in Missouri, Kentucky, and Washington states resulted from infection with agents resembling B. divergens based on the small subunit ribosomal RNA (SSU rRNA) gene and other factors.6–13 Morphologic similarity was noted in the wide angle between paired piroplasms and the frequent location of parasites at the internal margin of the erythrocyte in the accolé position.6–8

Most cases of human babesiosis caused by B. divergens have occurred in asplenic patients, thus resulting in a high fatality rate.12 The two cases reported in Kentucky and Missouri also occurred in asplenic patients, with the latter resulting in death.6,7 Serum from the Missouri patient reacted with B. divergens antigen in both indirect immunofluorescence and immunoprecipitation.6 In addition, the Missouri agent, designated MO-1, was not infective for gerbils, a standard criterion for identifying B. divergens.6

MO-1 and the causative agent in the Kentucky case are synonymous based on identical SSU rRNA gene sequences (GenBank accession nos. AY048113 and AY887131, respectively). The causative agent in the Kentucky case is reported to be nearly identical in SSU rRNA gene sequence to that of B. divergens (99.8% identity).7 Because homology of this order may be indicative of conspecificity, MO-1 and the Kentucky agent might be considered B. divergens.7 However, neither B. divergens nor its tick vector, Ixodes ricinus, is indigenous to the United States, and, indeed, both are considered exotic species.

Recently, a B. divergens–like parasite that shares identical SSU rRNA gene sequence with the Kentucky parasite was identified in eastern cottontail rabbits (Sylvilagus floridanus) and Ixodes dentatus ticks on Nantucket Island, Massachusetts.13 Although conspecificity of the eastern cottontail rabbit parasite with the Kentucky agent is supported by both morphology and molecular identity in the SSU rRNA gene,1,3,14 the relationship of this organism to B. divergens is not as clear. The B. divergens SSU rRNA gene sequence similarity to that of the rabbit parasite led to speculation that the rabbit parasite is B. divergens.13 Concern that B. divergens may be endemic in the United States and clarification of the potential of this parasite to infect native cattle led to this study. The study reported herein presents evidence supporting the conclusion that the Nantucket Babesia isolate is a species distinct from B. divergens based on host specificity.

MATERIALS AND METHODS

Parasites. The Babesia sp. isolated from eastern cottontail rabbits (Nantucket Babesia isolate, NR831) on Nantucket Island, Massachusetts, and the Purnell strain of B. divergens were previously described.13–17

The Nantucket Babesia isolate (NR831) was cultivated in vitro in eastern cottontail rabbit erythrocytes in HL-1 medium (BioWhittaker, Walkersville, MD) supplemented with 20% human serum, 2 mM l-glutamine (GibcoBRL, Grand Island, NY), and 200 U/mL penicillin, 200 μg/mL streptomycin, and 50 μg/mL Fungizone (antibiotic- antimycotic; GibcoBRL) as previously described.16 Babesia divergens Purnell was cultured as previously described in bovine erythrocytes but in
complete culture medium consisting of HL-1 medium supplemented with 20% normal adult bovine serum. The cultures were incubated at 37°C in a humidified atmosphere of 2% oxygen, 5% carbon dioxide, and 93% nitrogen and maintained following previously described protocols.\textsuperscript{14,16}

Cultures of NR831 and B. divergens in the sixth and eighth passages, respectively, were quantitated to produce $1 \times 10^5$ infected erythrocytes per dose. The doses were prepared in a final concentration of 10% polyvinylpyrrolidone 40 in Puck’s saline glucose with 10 g/L extra glucose and stored in liquid nitrogen. An aliquot of each isolate was successfully recovered in culture to confirm viability of the inoculum prior to use for experimental infections of calves described below. The frozen inocula were shipped on dry ice by overnight courier to the Animal Disease Research Unit, Agricultural Research Service, United States Department of Agriculture, Pullman, WA, and immediately stored in liquid nitrogen until used.

*Babesia divergens* Purnell and NR831 cultures were prepared as antigens for indirect immunofluorescence (IIF) tests as previously described.\textsuperscript{18}

**Experimental inoculations.** Seven 6- to 9-month-old Holstein-Friesian steers were obtained from a feedlot in Washington State. Each animal was confirmed Babesia-free after serologic screening using *B. divergens*-, *B. bovis*-, and *B. bigemina*-specific IIF. The steers were housed in a BL-2 facility and maintained as prescribed by the institutional biosafety and animal care committees (Washington State University, Pullman, WA) in keeping with National Institutes of Health guidelines.

Three of the steers were surgically splenectomized prior to the onset of the study. Principal animals, which each received an intravenous dose of $1 \times 10^5$ erythrocytes infected with NR831, included two splenectomized and three spleen-intact animals. One splenectomized and one spleen-intact animal served as controls, and each similarly received an intravenous dose of $1 \times 10^5$ erythrocytes infected with *B. divergens* Purnell. On Day 59 postinoculation (PI), each surviving principal and the spleen-intact control animal were challenged with an intravenous dose of $1 \times 10^5$ erythrocytes infected with *B. divergens* Purnell.

**Evaluation of infection.** Blood samples and rectal body temperature were obtained from each animal 17 days prior to inoculation and on Days 4-7, 9-14, 17, 19, 21, 26, and 33 PI. Blood samples collected into Alsever’s solution were centrifuged to pellet the cells, and the resulting cell pellet was washed by centrifugation as previously described.\textsuperscript{15} For each animal, packed washed erythrocytes (0.2 mL) were dispensed into 0.8 mL complete culture medium (HL-1 with 20% bovine serum as described above) in a well of a 24-well culture plate. In addition, cultures containing 0.1 mL packed erythrocytes from the principals and 0.1 mL washed packed human erythrocytes (Rockland Immunochernicals, Gilbertsville, PA) in 0.8 mL HL-1 medium with 20% human serum (Rockland Immunochernicals) were prepared. The cultures were incubated as above. Each day, 0.8 mL supernatant medium was removed from the cultures and replaced with fresh appropriate medium. Giemsa-stained erythrocyte smears were made daily from the cultures and examined microscopically at 1,000× under oil immersion to screen for the appearance of *Babesia* organisms. The cultures were maintained until parasites were detected or until 2 weeks after initiation. Cultures were not initiated from control animal blood samples after Day 11 PI.

**PCR.** A nested PCR protocol was used to amplify a small subunit ribosomal RNA (SSU rRNA) gene fragment common to both *B. divergens* and the Nantucket *Babesia* isolate. Genomic DNA was purified from 200 μL packed washed erythrocytes by a standard phenol/chloroform extraction protocol\textsuperscript{19} facilitated by use of Phase-lock gel tubes (Sigma-Aldrich, St. Louis, MO) and ethanol precipitated. The dried DNA pellets were resuspended in 20 μL TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and 1 μL of the suspension was used in a primary PCR with 1 pmol each forward primer A (5’-AACCTGGTGATATGCCAGT-3’) and reverse primer B (5’-GATCCTTCTGGAGAATCTCC-3’)\textsuperscript{20} in a 25 μL reaction volume (Advantage 2 PCR Kit, BD Biosciences, Palo Alto, CA) to amplify the SSU rRNA gene. The cycling protocol included an initial denaturation step at 96°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 10 seconds, annealing at 60°C for 10 seconds, and extension at 72°C for 2 minutes, with a final extension at 72°C for 10 minutes and hold at 4°C in a PCR Express or Sprint thermocycler (Hybaid, Waltham, MA).

Primers designed to specifically amplify a 1,026-bp fragment of the SSU rRNA gene common to both *B. divergens* and NR831 were used for nested PCR. The primary PCR products were diluted 1:20 in PCR grade water and 1 μL used as template in each reaction mixture as above, but with 1 pmol each primer srBdvF (3’-GGTGGTAATTTGAC-TAATGTCGAGA TTGC-5’) and srBdvR (3’-CCAAGCCGAAGC TGAAAGGCC-5’) and the initial denaturation reduced to 1 minute. The products were separated by electrophoresis through a 1% agarose gel, stained with ethidium bromide, and viewed by ultraviolet transillumination.

**RESULTS**

No evidence of infection was found in any of the principal animals inoculated with the Nantucket *Babesia* isolate (Figure 1). No clinical signs were noted, no detectable parasitemias were detected from stained blood films, all PCR assays were negative, and no parasites were detected in any of the cultures.

The splenectomized and spleen-intact control calves inocu-
lated with *B. divergens* subsequently developed clinical infections. Blood samples from the spleen-intact control animal were PCR positive from Day 4 through Day 18 PI, and *B. divergens* was detected in cultured blood samples collected on Day 5 onwards (Table 1). Parasites were first detected in blood films on Day 10 PI. A mild response to infection resulted with no evident hemoglobinuria or hemoglobinemia. The lowest hematocrit (25) was observed on Day 9 PI (Figure 1). Blood samples from the splenectomized control calf were PCR positive on Day 4 PI, negative on Day 5, and then positive for all subsequent samples (Table 1). Cultured blood samples confirmed infection from Day 4 onwards and parasitized erythrocytes were detected on Giemsa-stained blood films at Day 7 PI. Severe clinical signs of infection developed, which included lethargy and hemolytic anemia resulting in hemoglobinuria and hemoglobinemia. On Day 11 PI, in parallel with an elevated temperature, the hematocrit decreased to 17 and the parasitemia increased to 9%. The animal was humanely euthanized shortly thereafter.

After challenge inoculation with *B. divergens* Purnell, all five principal animals developed infections that were similar to the primary responses observed in the controls. The splenectomized and the spleen-intact principal calves were positive by PCR on Day 4 and Day 7 (not tested Days 5 or 6) postchallenge (PC), respectively (Table 2). Parasites were detected in blood smears from the two splenectomized principal animals on Day 8 and Day 9 and from each of the spleen-intact principal animals on Day 10 PC. Clinical disease resulting from challenge inoculation was severe in the two splenectomized principals, both presenting with hemoglobinuria and hemoglobinemia. Hematocrits decreased to 13 on Day 9 PC in one and 15 on Day 10 PC in the other (Figure 2). After detection of parasites from stained blood films, each animal was humanely euthanized. The control spleen-intact animal was completely protected from clinical manifestations upon challenge with *B. divergens* Purnell but remained positive by PCR (Tables 1 and 2).

Seroconversion was not detected by IIF in the principals after inoculation with the Nantucket *Babesia* isolate. Cross-reactivity to *B. divergens* Purnell and the Nantucket *Babesia* antigen was noted in the two control calves inoculated with *B. divergens* by Day 11 PI. The spleen-intact control animal maintained a titer that increased after the challenge inoculation with *B. divergens* Purnell. Each of the principal animals seroconverted after *B. divergens* Purnell challenge inoculation, with serologic cross-reactions between the *B. divergens* Purnell and Nantucket *Babesia* isolate antigens observed in the IIF test.

**DISCUSSION**

Since the first case of human babesiosis was diagnosed in the United States in 1966, hundreds of *B. microti* infections...
have been reported primarily in the northeastern and upper midwestern United States. Over the past decade, emergence of human babesiosis, caused by agents other than \( B. \) \( \text{microti} \) in geographic areas of the United States where the disease has not previously been known to occur, has raised awareness of this underreported, potentially fatal disease. The recognition of new agents in the United States capable of causing babesiosis in humans raises questions about their reservoir hosts and tick vectors. To date, these questions remain largely unanswered.

Reports of human babesiosis by parasites resembling \( B. \) \( \text{divergens} \) in Missouri, Kentucky, and Washington states are particularly perplexing. \( B. \) \( \text{divergens} \), a parasite of cattle that causes serious production losses, is also the primary cause of human babesiosis in Europe. Considered an exotic parasite, not only the human population but also resident cattle populations would be at risk if this parasite were established in the United States.

\( B. \) \( \text{divergens} \) species traditionally are defined based on parasite morphology, mammalian host, tick vector, and serologic characterization. More recently, molecular markers have added another dimension to identification of these organisms, and the use of DNA sequence data to differentiate Babesia species is now widely accepted. Although a number of genes have been investigated for their utility in parasite identification, to date the genetic databases hold more \( B. \) \( \text{divergens} \) spp. sequence data for the small subunit ribosomal RNA gene than any other single gene. SSU rRNA sequence comparisons have identified known \( B. \) \( \text{divergens} \) spp. in new mammalian hosts as well as parasites possessing previously unreported sequences that may represent newly discovered species. A drawback to this methodology is that there is no baseline sequence variation defined for any genetic marker for allopatric hemoprotozoan populations.

In the current case, a concern regarding whether a zoonotic \( B. \) \( \text{divergens} \)-like parasite in the United States is, in fact, \( B. \) \( \text{divergens} \) centers on the potential economic impact to the cattle industry in this country, in addition to the human health risk. Host specificity is a defining trait of \( B. \) \( \text{divergens} \) spp., thus, inoculation of cattle was undertaken to determine the infectivity of the U.S. parasite for this well-documented host of \( B. \) \( \text{divergens} \). We elected to test both spleen-intact and splenectomized calves to provide optimum conditions for parasite infectivity. Even \( B. \) \( \text{divergens} \) strains of low pathogenicity may be tested for host specificity using splenectomized calves because they are less efficient in clearing the parasites and thus more susceptible to infection.

Parallel inocula of \( B. \) \( \text{divergens} \) and the Nantucket \( B. \) \( \text{babesia} \) isolate (conspecific with the U.S. human agent) were prepared from parasite cultures at similar passage levels (passes 8 and 6, respectively). Early passages were used because culture attenuation has been reported for some \( B. \) \( \text{divergens} \) spp., and \( B. \) \( \text{divergens} \) has been reported variously as attenuated over time in culture or as unaffected by long-term passage in vitro. To preclude the possibility of such effects, parasites from very early passages were used for both the primary inoculation and challenge doses. No clinical disease ensued after inoculation of either principal spleen-intact or splenectomized calves with the Nantucket \( B. \) \( \text{babesia} \) isolate. Furthermore, parasites were not detected by either PCR or culture in any of the principals at any time. Finally, primary inoculation with the Nantucket \( B. \) \( \text{babesia} \) isolate provided no protection upon subsequent challenge inoculation with \( B. \) \( \text{divergens} \). Both the three spleen-intact and two splenectomized principal animals became infected with \( B. \) \( \text{divergens} \), and severe clinical disease occurred in the two splenectomized animals. Both culture and PCR confirmed infection and identified the infections 3 to 5 days sooner than stained blood films. In contrast, the spleen-intact \( B. \) \( \text{divergens} \) Purnell control calf was completely protected from clinical disease upon homologous challenge.

Molecular characterization based on the SSU rRNA gene indicates that the Nantucket Island parasite and that found in man in Kentucky are likely the same organism. Furthermore, it is now known that the Nantucket Island, Kentucky, and Missouri parasites share identical SSU rRNA gene sequences (GenBank accession nos. AY144688, AY887132, and AY904043; AY887131; and AY048113, respectively). MO-1 also failed to infect calves, which contributed to the conclusion that MO-1 was not \( B. \) \( \text{divergens} \). Thus, it is also likely that MO-1 is conspecific with the Nantucket Island \( B. \) \( \text{babesia} \). It appears that the eastern cottontail rabbit, which is found throughout the United States, may be a natural reservoir host for this parasite. Molecular evidence shows that \( Ixodes \) \( \text{dentatus} \), which feeds primarily on rabbits, may serve as the tick vector for this \( B. \) \( \text{babesia} \) sp.

On the other hand, the \( B. \) \( \text{divergens} \) SSU rRNA gene sequence differs slightly from that of the Nantucket, Kentucky, and MO-1 isolates, suggesting that these North American iso-
lates may not be conspecific with the European agent of bovine and human babesiosis, nor is this parasite the same as the B. divergens–like parasite isolations from wild ungulates in North America.\textsuperscript{15,32–35} Those characterized by SSU rRNA gene sequence analysis were identified as B. odocoilei\textsuperscript{14,15} or as divergent from, but closely related to, B. divergens.\textsuperscript{36} Phylogenetic studies based on the SSU rRNA gene show that B. odocoilei is closely related to B. divergens (Purnell strain = type species) (20–30 base pair difference), and these two species branch together in the true Babesia clade.\textsuperscript{14,36,37}

Multiple rRNA transcriptional units with variable SSU rRNA gene sequences are reported for the bovine parasites B. bovis and B. bigemina, demonstrating that SSU rRNA gene variability does exist within some Babesia species.\textsuperscript{38,39} On the other hand, identical SSU rRNA gene sequences have been found in the aforementioned isolates of B. odocoilei, whether isolated from white-tailed deer,\textsuperscript{40} caribou,\textsuperscript{32} elk,\textsuperscript{15,33} musk oxen, or bighorn sheep.\textsuperscript{37} The biology of B. bovis, which is a bovine-specific parasite, differs from that of B. divergens or B. odocoilei, which infect diverse host species. However, all three may be vectored by Ixodes spp. ticks.\textsuperscript{40} Thus, perhaps various Babesia spp. may be similar or dissimilar from each other in rRNA transcriptional unit make-up as well. Moreover, the degree of intraspecies SSU rRNA sequence variation may differ as well, depending on the Babesia species. If so, then absolute boundaries cannot be drawn across this genus for species delineation based on the SSU rRNA gene.

This study clearly shows that despite morphologic, serological, clinical course of human disease, at-risk human factors (i.e., splenectomy), tick vector (Ixodes spp.), and genetic similarities, B. divergens and the U.S. Babesia sp. are not conspecific as they do not share the bovine host. In the case of these two parasites, a three-base difference in the SSU rRNA gene sequence is sufficient to molecularly distinguish the two species.

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