In Vitro Cultivation of a Zoonotic *Babesia* sp. Isolated from Eastern Cottontail Rabbits (*Sylvilagus floridanus*) on Nantucket Island, Massachusetts

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A *Babesia* sp. found in eastern cottontail rabbits (*Sylvilagus floridanus*) on Nantucket Island, Massachusetts, is the same organism that caused human babesiosis in Missouri and Kentucky, on the basis of morphology and identical small-subunit rRNA (SSU rRNA) gene sequences. Continuous cultures of the rabbit parasite were established from infected blood samples collected from two cottontail rabbits live-trapped on Nantucket Island. HL-1 medium or minimal essential medium alpha medium supplemented with 20% human serum best supported in vitro propagation of the parasite in human or cottontail erythrocytes, respectively. Parasite growth was not sustained in domestic-rabbit erythrocytes or in medium supplemented with domestic-rabbit serum. The cultured parasites were morphologically indistinguishable from the Kentucky human isolate. Transmission electron microscopy revealed similar fine structures of the parasite regardless of the host erythrocyte utilized in the cultures. Two continuous lines of the zoonotic *Babesia* sp. were established and confirmed to share identical SSU rRNA gene sequences with each other and with the Missouri and Kentucky human *Babesia* isolates.

Human babesiosis is most commonly caused by the tick-borne hemoproteoans *Babesia microti* in the United States and *Babesia divergens* in Europe. Although the latter organism seldom causes clinical disease in immunocompetent individuals, fatal infections often result in immunocompromised persons. Neither *B. divergens* nor its tick vector is endemic in the United States, but two cases of acute human babesiosis in Missouri and Kentucky were attributed to *B. divergens*-like parasites that share similar pathologies (4, 9) and identical small-subunit rRNA (SSU rRNA) gene sequences (accession numbers AY048113 and AY887131, respectively). The SSU rRNA gene sequence is nearly identical to that of *B. divergens* (accession numbers U16370 [11] and AY046576 [12]). A third similar infection was recently reported from Washington State (10).

Molecular evidence indicates that the organism identified in the Missouri and Kentucky human cases is also found in eastern cottontail rabbit (*Sylvilagus floridanus*) populations on Nantucket Island, Massachusetts (8), which suggests that these animals may be reservoir hosts for the parasite. At present, the geographic range of the eastern cottontail extends from Canada to South America, including the central United States. Eastern cottontail rabbits are not native to Massachusetts, having been introduced by sportsman clubs during the early 1900s (3). Thousands of rabbits were transported from mid-western states, including Missouri and Kentucky, to Massachu-

setts and released for game hunting. The *S. floridanus* cottontails became established, displacing the native New England cottontail (*Sylvilagus transitionalis*) populations. The rabbit *Babesia* sp. was likely introduced along with the host cottontail rabbit (CT), in the same way that tularemia was brought into Massachusetts (3, 5). Coincidentally, the patient in the Kentucky case hunted and dressed cottontail rabbits prior to the onset of clinical signs of babesiosis (4).

The characterization of numerous *Babesia* species has been facilitated by establishing laboratory sources of the isolate, either by subinoculation of an isolate into a suitable laboratory animal host or by in vitro cultivation of the parasite. Attempts to infect laboratory animals or to establish cultures were unsuccessful in the Missouri case (9), and neither technique was reported in the Kentucky case (4). The discovery that cottontail rabbits on Nantucket Island harbor these parasites provides a source of inoculum for culture initiation. Continuous cultures of these parasites in both human and cottontail rabbit erythrocytes were established from infected cottontail rabbit blood and are described herein.

MATERIALS AND METHODS

Parasites. During a 2-year period, 2002 to 2003, blood samples were collected from the same population of eastern cottontail rabbits (*S. floridanus*) previously studied on Nantucket Island, Massachusetts (8). Blood samples were collected in 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. Giemsa-stained blood smears were examined, and the samples were tested for the presence of babesias by a PCR, as previously described (8). Samples were shipped on ice overnight to Texas A&M University, College Station, Texas.

In vitro culture. Uninfected donor blood and cottontail rabbit blood samples positive by PCR for babesias were prepared similarly prior to use. The blood was

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TABLE 1. Culture conditions, culture levels (primary or passage) tested, and outcomes for cottontail rabbit *Babesia* sp. cultures

Complete medium ^f	Basal medium	Serum	Supplement		RBC source	Culture level(s) tested ^h	Continuous culture
			HB101 ^a	H-T ^b			
HLHS	HL-1 ^c	20% Human ^d	No	No	Human ^d	1°; 1st pass	Yes
HLRS	HL-1	20% Rabbit ^e	No	No	Rabbit ^d	1°; 2nd pass	No
HL+HS	HL-1	20% Human	Yes	Yes	CT	1°	No
					CT	1°; 1st pass	Yes
UC+HS	UltraCulture ^c	20% Human	Yes	Yes	Human	3rd pass	Yes
AlphaHS	MEM Alpha	20% Human	No	No	CT	1°	ID ^f
					CT	1°; 1st pass	Yes
					Human	1°	ID
					Human	2nd pass	No
					Rabbit	1°; 2nd pass	No
AlphaRS	MEM Alpha	40% Rabbit	No	No	CT	1°	No
AlphaFBS	MEM Alpha	20% FBS ^g	No	No	CT	1°	No

^a Irvine Scientific, Irvine, Calif.^b Hypoxanthine-thymidine; Gibco (14).^c BioWhittaker, Walkersville, MD.^d Rockland Immunochemicals, Gilbertsville, Pa.^e New Zealand White domestic rabbit serum; Sigma Chemical Co., St. Louis, Mo.^f ID, insufficient data due to pooling of cultures prior to first passage.^g Hyclone Laboratories, Inc., Logan, Utah.^h 1°, primary culture.

ⁱ HLHS, HL-1 medium supplemented with human serum; HLRS, HL-1 medium supplemented with rabbit serum; HL+HS, HL-1 medium supplemented with human serum, HB101, and H-T; UC+HS, UltraCulture supplemented with human serum, HB101, and HT; AlphaRS, MEM Alpha medium supplemented with rabbit serum; AlphaFBS, MEM Alpha medium supplemented with fetal bovine serum.

centrifuged at $500 \times g$ for 20 min to pellet the cells, and the plasma and buffy layer were then discarded. The red blood cells (RBC) were washed three times in 5 volumes of RPMI 1640 medium by centrifugation at $500 \times g$ for 20 min each wash, with removal of the buffy layer at each wash. After the final wash, the supernatant was removed and the RBC were used as packed cells. Donor RBC not used at this time were stored in RPMI 1640 medium and used within 5 days. PCR-negative cottontail rabbit blood, human blood (type O, Rh+) (Rockland Immunochemicals, Gilbertsville, Pa.), and domestic New Zealand White rabbit blood (Rockland Immunochemicals) stored in Alsever's solution at 4°C remained usable as donor RBC for a maximum of 3 weeks.

Cultures were initiated in 24-well culture plates in media formulated as shown in Table 1, with each medium also supplemented with 1 mM L-glutamine, 200 µg/ml streptomycin, 200 U/ml penicillin, 50 µg/ml amphotericin B (Fungizone, antibiotic-antimycotic; Gibco BRL, Grand Island, N.Y.), and 100 µg/ml gentamicin (Gibco). Primary parasite cultures from Nantucket Island cottontail rabbit 831 (NR831) contained 250 µl RBC per well in 1.0 ml of medium (Table 1; Fig. 1). Primary parasite cultures from Nantucket Island cottontail rabbit 774

(NR774) contained 200 µl RBC per well in 800 µl of medium (Table 1). A single culture well containing 200 µl RBC in 800 µl minimum essential medium alpha medium (MEM Alpha; Gibco) was initiated from cottontail rabbit 831 blood stored at 4°C for 3 weeks. The cultures were incubated at 37°C in a humidified modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, Calif.) in a gas mixture of 5% carbon dioxide, 2% oxygen, and 93% nitrogen. The cultures were moved to a humidified atmosphere of 5% carbon dioxide in air after establishment.

Cultures were fed daily by removing 800 µl (1 ml culture volume) or 1 ml (1.25 ml culture volume) medium without disturbing the settled RBC layer and replacing this with 800 µl or 1 ml appropriate fresh medium, respectively. Additional packed RBC (50 µl) were added every 3 to 5 days as the RBC layer thinned due to erythrocytolysis.

The first passage was performed at a split ratio of 1:2. At subculture, the cultures were fed as above, the RBC were resuspended, and one-half volume of the RBC suspension was transferred to a new well. The volumes of both wells were brought to the original culture volume of 1 ml or 1.25 ml by the addition of

FIG. 1. Flow chart depicting the various conditions tested for the cottontail rabbit 831 parasite cultures. Each box shows the culture level, complete medium, and RBC source, followed by whether the culture was established or terminated. See Table 1, footnote i, for complete-media formulations. NZ, New Zealand.

FIG. 2. Various morphological forms of the cottontail rabbit parasite (top row) cultured in cottontail rabbit erythrocytes are shown, compared to similar parasite forms in a blood smear from the human case in Kentucky (bottom row). Paired pyriforms (first panel of each row) are the diagnostic form. Giemsa stain. Scale bar, 5 μ m.

medium and 100 μ l uninfected-donor packed RBC. Subsequent subcultures were similarly performed at split ratios of 1:2, 1:4, or 1:5, with the addition of 100 μ l packed donor RBC to each culture. After establishment, the cultures were routinely split at a 1:5 ratio.

Parasite growth in early passages was monitored by spotting 0.1 μ l of culture RBC onto a microscope slide. The spot was then air dried, fixed twice with methanol, and stained with Giemsa (Accustain; Sigma, St Louis, Mo.). The parasitized erythrocytes in the entire spot were tallied at a $\times 500$ magnification under oil, and the percent parasitemia was calculated. After establishment, the percent parasitemia was calculated by enumeration from 1,000 erythrocytes on Giemsa-stained RBC smears at $\times 1000$ under oil.

The parasite cultures were cryopreserved and subsequently recovered as previously described, except in a final concentration of 10% polyvinylpyrrolidone 40 as the cryoprotectant (Sigma) (13).

SSU rRNA gene sequence analysis. DNA was purified from NR831- and NR774-cultured parasites (14th passage and 7th passage, respectively) by a standard phenol-chloroform extraction procedure (21). The SSU rRNA gene was amplified, cloned, and sequenced using previously described methods (14). The resulting sequences were aligned using Sequencher 3.11 software (Gene Codes Corporation, Inc., Ann Arbor, Mich.), and individual sequences were subjected to BLAST similarity searches (National Center for Biotechnology Information, National Institutes of Health; <http://www.ncbi.nlm.nih.gov/BLAST/>) (2). The sequences were directly compared with the SSU rRNA gene sequences from the parasite in the human case in Kentucky and *B. microti* (Ruebush strain, accession number U09833 [1]) (Genestream Resource Center; <http://www2.igh.cnrs.fr/bin/lalign-guess.cgi>) (4, 20).

Transmission electron microscopy. NR831 parasite cultures in cottontail rabbit (passage 11) or human (Hu) RBC (passage 10) grown in HL-1 medium with human serum, HB101 supplement, and H-T added (HL+HS) were processed for transmission electron microscopy (TEM). The medium overlaying the RBC layer was removed, and 100 μ l of concentrated RBC was transferred, with gentle mixing, to 10 ml of 1% isosmotic glutaraldehyde (300 mOsm/kg) buffered with sodium phosphate buffer (6). Mixing was continued for 1 h at 20 rpm on a tube rotator (Dyna, Inc., New Hyde Park, N.Y.) at 25°C. The cells were postfixed in 1% osmium tetroxide in 100 mM phosphate buffer, pH 7.4, with 100 mM sucrose for 90 min at 4°C, followed by distilled water washes (six changes over 60 min), and then poststained in 1% uranyl acetate for 60 min at 4°C. The cells were washed twice in distilled water and then pelleted in 2% agar, dehydrated in an ethanol series followed by acetone, and embedded in epoxy resin for examination by TEM.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study were deposited in the GenBank database under accession numbers AY887131 (Kentucky *Babesia* sp.), AY887132 (NR831 *Babesia* sp.), and AY904043 (NR774 *Babesia* sp.).

RESULTS

Parasites. During a 2-year period, 2002 to 2003, 104 eastern cottontail rabbit blood samples were collected. For the year

2002, nine samples were positive for babesias by PCR (88 total, 10.2% prevalence, 95% confidence interval [4.8, 18.5]), and for the year 2003, three samples were positive (26 total, 11.5% prevalence, 95% confidence interval [2.4, 30.2]). Although no parasites were detected on Giemsa-stained blood films, continuous parasite cultures were successfully established from the last two rabbits, 831 and 774, trapped in 2003. The rabbit parasite was morphologically indistinguishable from the causative agent of human babesiosis reported in Kentucky (Fig. 2) (4).

Continuous cultures. Cottontail rabbit parasite primary cultures were successfully propagated in HLHS or MEM Alpha supplemented with human serum (AlphaHS) (Fig. 1). Parasites were visible in Giemsa-stained RBC by day 6 in cultures initiated from 831, stored 831, and 774 blood samples in both media.

The success or failure of various medium and erythrocyte combinations tested for NR831 parasite cultures is depicted in Fig. 1. The optimal conditions from passage 1 onward were either donor Hu RBC in HLHS or CT RBC in AlphaHS. Passages 1 and 2 were performed on days 8 and 12, respectively, as a parasitemia of approximately 0.15% was achieved. Passages 3 to 6 were done at 2- to 5-day intervals, depending on parasite growth, as parasitemias of 0.15 to 0.5% were reached. Continuous cultures resulted when the parasites were subcultured into HL+HS and Hu RBC from either HLHS- or AlphaHS-supplemented cultures at passage 4 or 5 (Table 1). Throughout culture establishment, the intervals between subcultures and the split ratios employed varied depending on parasite proliferation (Table 2).

NR774 parasite cultures were initiated in HL+HS, AlphaHS, UltraCulture medium (BioWhittaker) supplemented with human serum, or MEM Alpha supplemented with fetal bovine serum (FBS) (Table 1). At 12 days, the cultures in human serum were combined into a single well in HL+HS in an attempt to bolster parasite growth. Passages 1 and 2 (1:2 split ratio) were done on day 18 and 26, respectively, into Hu RBC or CT RBC in HLHS. The parasites subcultured into Hu RBC at passage 2 survived, but passage 1 parasites did not. From passage 2 onward, either Hu RBC or CT RBC in HL+HS supported parasite proliferation. After passage 5, the

TABLE 2. Comparison of cottontail rabbit 831 parasites cultivated in human or cottontail rabbit erythrocytes

Medium, RBC source	Day	Passage	% Parasitemia	Subculture split ratio
HLHS, human	12	2	0.18	1:2
	16	3	0.16	1:2
	18	4	0.17	1:2
	23	5	0.29	1:2
	26	6	0.29	1:2
	28	7	0.41	1:2
AlphaHS, cottontail	12	2	0.52	1:4
	14	3	0.50	1:4
	18	4	0.26	1:2
	20	5	0.47	1:4
	22	6	0.50	1:4
	24	7	0.49	1:4

cultures were routinely subcultured 1:5 every 4 to 7 days. The parasitemias ranged from 4 to 9% at subculture in CT RBC and from 2 to 4% in Hu RBC.

Long-term growth of the parasite was not supported by AlphaHS combined with Hu RBC or by domestic-rabbit RBC or serum regardless of the medium used (Table 1). The single attempt to utilize alpha medium supplemented with FBS was not successful.

Cottontail rabbit 831 infected blood stored at 4°C for 3 weeks yielded a viable culture in AlphaHS. The parasites were maintained in CT RBC for two passages and then successfully subcultured into Hu RBC and HLHS at passage 3. At passage 3, cultures in HL+HS and Hu RBC were incubated in either 5% carbon dioxide in air or low oxygen tension, humidified, as described above. The parasites thrived under both conditions, and the cultures were routinely split at a 1:4 ratio every 4 to 5 days.

Cryopreserved parasite cultures in human or cottontail rabbit RBC were successfully recovered into HL+HS with Hu or CT RBC, respectively (not shown).

SSU rRNA gene sequence analysis. SSU rRNA genes were successfully amplified, cloned, and sequenced from both NR831- and NR774-cultured parasites. An alignment of the obtained sequences (graphic alignment not shown) revealed that the NR831 culture and the NR774 culture possess identical SSU rRNA sequences (accession numbers AY887132 and AY904043, respectively) of 1,724 bp, which are also identical to those of the agents of human babesiosis in Kentucky (accession number AY887131) and Missouri (accession number AY048113) (BLAST similarity search) (Fig. 3). The sequence shares 99.8% identity, differing in three base positions, with that of *B. divergens* from cattle (accession numbers U16370 [10] and AY046576 [11]) (Fig. 3). The sequence also differs in three base positions from that of a reindeer *Babesia* isolate (accession number AY098643 [17]) and in five base positions (99.7% identity) from that of a *B. divergens*-like parasite reported from a human case of babesiosis in Washington State (accession number AY274114 [12]). The *B. microti* (accession number U09833) SSU rRNA gene shares 88.2% identity with the sequence from the Nantucket Island cottontail rabbit and the Kentucky isolates.

TEM. Parasites in cottontail erythrocyte or human erythrocyte cultures show similar fine structures that are consistent with that previously reported for *B. divergens* (7). Merozoites are bound by an outer limiting membrane exterior to a two-layer inner membrane complex that extends along the length of the organism (Fig. 4). A large double-membrane-bound nucleus, double-membrane-bound mitochondrion-like structures, and an endoplasmic reticulum throughout the cytoplasm, often with associated ribosomes, are evident. Rhoptries located at the end of the parasite form an apical complex with the inner membrane complex.

DISCUSSION

Two cases of human babesiosis attributed to a *B. divergens*-like agent in the south central region of the United States resulted in a fatal case in Missouri and a nonfatal but acute case in Kentucky (4, 9). Additionally, an acute case of babesiosis also attributed to a *B. divergens*-like agent occurred in Washington State (10). The organism in all three cases was described as morphologically and molecularly similar to *B. divergens*. Further characterization of this parasite has been hampered by the small amount of original sample (4) and no additional source of parasites. Recently, a *Babesia* sp. of eastern cottontail rabbits was reported to have an SSU rRNA gene sequence identical to that of the Kentucky human agent (8). The current study confirms this finding and, further, demonstrates that the Missouri isolate SSU rRNA gene sequence is also identical to that of the rabbit parasite. This study also shows the rabbit parasite to be morphologically indistinguishable from the Kentucky human parasite. Although eastern cottontails potentially provide a source of parasites, the circulating parasitemias are extremely low (8); other experimental hosts have not been reported. The successful establishment of continuous in vitro cultures of this organism is not only the first culture isolation and establishment of a zoonotic *Babesia* sp. from its natural host in the United States but also an important step toward further studies of this organism.

Continuous cultures of the cottontail rabbit *Babesia* sp. were established in both cottontail rabbit and human erythrocytes with medium supplemented with human serum. Of the media tested for their abilities to support parasite growth, HL-1 medium best supported the parasite over extended culture periods, although MEM Alpha produced the highest parasitemias in cottontail erythrocyte cultures during early passages. In this study, neither domestic-rabbit serum nor FBS supplementation sustained parasite growth. Inasmuch as previous attempts in this laboratory to initiate primary cultures from infected cottontail rabbit blood in HL-1 medium supplemented with FBS were not successful (unpublished results) and a single attempt to initiate a culture in MEM Alpha supplemented with FBS in the current study failed, we concentrated our efforts toward finding a suitable serum alternative. Hence, although supplementation with heat-inactivated FBS for *B. divergens* primary cultures has been reported (19), this option was not explored in this study.

Babesia spp. are often cultivated in medium containing autologous serum and RBC of the vertebrate host, modeled after the culture system introduced by Levy and Ristic (18). This strategy was not possible in the current study due to the lack of

KY/NR	<i>Babesia</i>	AACCTGGTTG	ATCCTGCCAGTAGTCATATG	CTTGTCTTAAAGATTAAGCC	ATGCATGTCTAAGTACAAAC	70
MO-1		AACCTGGTTG	ATCCTGCCAGTAGTCATATG	CTTGTCTTAAAGATTAAGCC	ATGCATGTCTAAGTACAAAC	
Bdiv	<i>B. divergens</i>	AACCTGGTTG	ATCCTGCCAGTAGTCATATG	CTTGTCTTAAAGATTAAGCC	ATGCATGTCTAAGTACAAAC	
KY/NR		TTTTTACGGTGAAACTGCGA	ATGGCTCATTACAACAGTTA	TAGTTTCTTTGGTATTCGTT	TTCCATGGATAACCGTGCTA	150
MO-1		TTTTTACGGTGAAACTGCGA	ATGGCTCATTACAACAGTTA	TAGTTTCTTTGGTATTCGTT	TTCCATGGATAACCGTGCTA	
Bdiv		TTTTTACGGTGAAACTGCGA	ATGGCTCATTACAACAGTTA	TAGTTTCTTTGGTATTCGTT	TTCCATGGATAACCGTGCTA	
KY/NR		ATTGTAGGGCTAATACAAGT	TCGAGGCCTTTTGGCGGCGT	TTATTAGTTCTAAAACCATC	CCTTTTGGTTTTTCGGTGATT	230
MO-1		ATTGTAGGGCTAATACAAGT	TCGAGGCCTTTTGGCGGCGT	TTATTAGTTCTAAAACCATC	CCTTTTGGTTTTTCGGTGATT	
Bdiv		ATTGTAGGGCTAATACAAGT	TCGAGGCCTTTTGGCGGCGT	TTATTAGTTCTAAAACCATC	CCTTTTGGTTTTTCGGTGATT	
KY/NR		CATAATAAACTTGCGAATCG	CAATTTTTTGGCATGGACCA	TTCAAGTTTCTGACCCATCA	GCTTGACGGTAGGGTATTGG	310
MO-1		CATAATAAACTTGCGAATCG	CAATTTTTTGGCATGGACCA	TTCAAGTTTCTGACCCATCA	GCTTGACGGTAGGGTATTGG	
Bdiv		CATAATAAACTTGCGAATCG	CAATTTTTTGGCATGGACCA	TTCAAGTTTCTGACCCATCA	GCTTGACGGTAGGGTATTGG	
KY/NR		CCTACCGAGGCAGCAACGGG	TAACGGGGAATTAGGGTTTCG	ATTCCGGAGAGGGAGCCTGA	GAAACGGCTACCACATCCAA	390
MO-1		CCTACCGAGGCAGCAACGGG	TAACGGGGAATTAGGGTTTCG	ATTCCGGAGAGGGAGCCTGA	GAAACGGCTACCACATCCAA	
Bdiv		CCTACCGAGGCAGCAACGGG	TAACGGGGAATTAGGGTTTCG	ATTCCGGAGAGGGAGCCTGA	GAAACGGCTACCACATCCAA	
KY/NR		GGAAGGCAGCAGGCGCGCAA	ATTACCCAATCCTGACACAG	GGAGGTAGTGACAAGAAATA	ACAATGCAGGGCAATTGTCT	470
MO-1		GGAAGGCAGCAGGCGCGCAA	ATTACCCAATCCTGACACAG	GGAGGTAGTGACAAGAAATA	ACAATGCAGGGCAATTGTCT	
Bdiv		GGAAGGCAGCAGGCGCGCAA	ATTACCCAATCCTGACACAG	GGAGGTAGTGACAAGAAATA	ACAATGCAGGGCAATTGTCT	
KY/NR		TGTAATTGGAATGATGGTGA	CCTAAACCCTCACCAGAGTA	ACAATTGGAGGGCAAGTCTG	GTGCCAGCAGCCGCGGTAAT	550
MO-1		TGTAATTGGAATGATGGTGA	CCTAAACCCTCACCAGAGTA	ACAATTGGAGGGCAAGTCTG	GTGCCAGCAGCCGCGGTAAT	
Bdiv		TGTAATTGGAATGATGGTGA	CCTAAACCCTCACCAGAGTA	ACAATTGGAGGGCAAGTCTG	GTGCCAGCAGCCGCGGTAAT	
KY/NR		TCCAGCTCCAATAGCGTATA	TAAACTTGTTCAGTTAAA	AAGCTCGTAGTTGAATTTTT	GCGTGGTGTAAATATTGACT	630
MO-1		TCCAGCTCCAATAGCGTATA	TAAACTTGTTCAGTTAAA	AAGCTCGTAGTTGAATTTTT	GCGTGGTGTAAATATTGACT	
Bdiv		TCCAGCTCCAATAGCGTATA	TAAACTTGTTCAGTTAAA	AAGCTCGTAGTTGAATTTTT	GCGTGGTGTAAATATTGACT	
KY/NR		GATGTCGAGATTGCACTTCG	CTTTTGGGATTATCCCTTT	TTACTTTGAGAAAATTAGAG	TGTTTCAAGCAGACTTTTGT	710
MO-1		GATGTCGAGATTGCACTTCG	CTTTTGGGATTATCCCTTT	TTACTTTGAGAAAATTAGAG	TGTTTCAAGCAGACTTTTGT	
Bdiv		<u>AATGTCGAGATTGCACTTCG</u>	CTTTTGGGATTATCCCTTT	TTACTTTGAGAAAATTAGAG	TGTTTCAAGCAGACTTTTGT	
KY/NR		CTTGAATACTTCAGCATGGA	ATAATAGAGTAGGACTTTGG	TTCTATTTTGTGGTTTGTG	AACCTTAGTAATGGTTAATA	790
MO-1		CTTGAATACTTCAGCATGGA	ATAATAGAGTAGGACTTTGG	TTCTATTTTGTGGTTTGTG	AACCTTAGTAATGGTTAATA	
Bdiv		CTTGAATACTTCAGCATGGA	ATAATAGAGTAGGACTTTGG	TTCTATTTTGTGGTTTGTG	AACCTTAGTAATGGTTAATA	
KY/NR		GGAACGGTTGGGGGCATTTCG	TATTTAACAGTCAGAGGTGA	AATTCTTAGATTGTGTTAAAG	ACGAACTACTGCGAAAGCAT	870
MO-1		GGAACGGTTGGGGGCATTTCG	TATTTAACAGTCAGAGGTGA	AATTCTTAGATTGTGTTAAAG	ACGAACTACTGCGAAAGCAT	
Bdiv		GGAACGGTTGGGGGCATTTCG	TATTTAAC <u>T</u> GTCAGAGGTGA	AATTCTTAGATTGTGTTAAAG	ACGAACTACTGCGAAAGCAT	
KY/NR		TTGCCAAGGACGTTTTTCATT	AATCAAGAACGAAAGTTAGG	GGATCGAAGACGATCAGATA	CCGTCGTAGTCCTAACCAT	950
MO-1		TTGCCAAGGACGTTTTTCATT	AATCAAGAACGAAAGTTAGG	GGATCGAAGACGATCAGATA	CCGTCGTAGTCCTAACCAT	
Bd		TTGCCAAGGACGTTTTTCATT	AATCAAGAACGAAAGTTAGG	GGATCGAAGACGATCAGATA	CCGTCGTAGTCCTAACCAT	
KY/NR		AACTATGCCGACTAGGGATT	GGAGGTGCTCATTTTCCGA	CTCCTTCAGCACCTTGAGAG	AAATCAAAGTCTTTGGGTTT	1030
MO-1		AACTATGCCGACTAGGGATT	GGAGGTGCTCATTTTCCGA	CTCCTTCAGCACCTTGAGAG	AAATCAAAGTCTTTGGGTTT	
Bdiv		AACTATGCCGACTAGGGATT	GGAGGTGCTCATTTTCCGA	CTCCTTCAGCACCTTGAGAG	AAATCAAAGTCTTTGGGTTT	
KY/NR		TGGGGGGAGTATGGTCGCAA	GGCTGAAACTTAAAGGAATT	GACGGAAGGGCACCACCAGG	CGTGGAGCCTGCGGCTTAAT	1110
MO-1		TGGGGGGAGTATGGTCGCAA	GGCTGAAACTTAAAGGAATT	GACGGAAGGGCACCACCAGG	CGTGGAGCCTGCGGCTTAAT	
Bdiv		TGGGGGGAGTATGGTCGCAA	GGCTGAAACTTAAAGGAATT	GACGGAAGGGCACCACCAGG	CGTGGAGCCTGCGGCTTAAT	
KY/NR		TTGACTCAACACGGGGAAAC	TCACCAGGTCCAGACAAATGT	TAGGATTGACAGATTGATAG	CTCTTTCTTGATTCTTTGGG	1190
MO-1		TTGACTCAACACGGGGAAAC	TCACCAGGTCCAGACAAATGT	TAGGATTGACAGATTGATAG	CTCTTTCTTGATTCTTTGGG	
Bdiv		TTGACTCAACACGGGGAAAC	TCACCAGGTCCAGACAAATGT	TAGGATTGACAGATTGATAG	CTCTTTCTTGATTCTTTGGG	
KY/NR		TGGTGGTGCATGGCCGTTCT	TAGTTGGTGGAGTGATTGT	CTGGTTAATTCGGTTAACGA	ACGAGACCTTAACCTGCTAA	1270
MO-1		TGGTGGTGCATGGCCGTTCT	TAGTTGGTGGAGTGATTGT	CTGGTTAATTCGGTTAACGA	ACGAGACCTTAACCTGCTAA	
Bdiv		TGGTGGTGCATGGCCGTTCT	TAGTTGGTGGAGTGATTGT	CTGGTTAATTCGGTTAACGA	ACGAGACCTTAACCTGCTAA	
KY/NR		CTAGTGTCCTGTAAGAGGTT	CGTCCGTTACGGTTTGCTTC	TTAGAGGGACTTTGCGGCTC	TAAGCCGCAAGGAAGTTTAA	1350
MO-1		CTAGTGTCCTGTAAGAGGTT	CGTCCGTTACGGTTTGCTTC	TTAGAGGGACTTTGCGGCTC	TAAGCCGCAAGGAAGTTTAA	
Bdiv		CTAGTGTCCTGTAAGAGGTT	CGTCCGTTACGGTTTGCTTC	TTAGAGGGACTTTGCGGCTC	TAAGCCGCAAGGAAGTTTAA	
KY/NR		GGCAATAACAGGTCTGTGAT	GCCCTTAGATGTCCTGGGCT	GCACGCGCGCTACACTGATG	CATTTCATCGAGTTTATCCC	1430
MO-1		GGCAATAACAGGTCTGTGAT	GCCCTTAGATGTCCTGGGCT	GCACGCGCGCTACACTGATG	CATTTCATCGAGTTTATCCC	
Bdiv		GGCAATAACAGGTCTGTGAT	GCCCTTAGATGTCCTGGGCT	GCACGCGCGCTACACTGATG	CATTTCATCGAGTTTATCCC	
KY/NR		TTCCCGAAAGGGCTGGGTAA	TCTTTAGTATGCATCGTGAC	GGGGATTGATTTTGCATTT	CTAAATCATGAACGAGGAAT	1510
MO-1		TTCCCGAAAGGGCTGGGTAA	TCTTTAGTATGCATCGTGAC	GGGGATTGATTTTGCATTT	CTAAATCATGAACGAGGAAT	
Bdiv		TTCCCGAAAGGGCTGGGTAA	TCTTTAGTATGCATCGTGAC	GGGGATTGATTTTGCATTT	CTAAATCATGAACGAGGAAT	
KY/NR		GCCTAGTATGCGCAAGTCAT	CAGCTTGTGCAGATTACGTC	CCTGCCCTTTGTACACACCG	CCCGTCGCTCCTACCGATCG	1590
MO-1		GCCTAGTATGCGCAAGTCAT	CAGCTTGTGCAGATTACGTC	CCTGCCCTTTGTACACACCG	CCCGTCGCTCCTACCGATCG	
Bdiv		GCCTAGTATGCGCAAGTCAT	CAGCTTGTGCAGATTACGTC	CCTGCCCTTTGTACACACCG	CCCGTCGCTCCTACCGATCG	
KY/NR		AGTGATCCGGTGAATTATTC	GGACCGTGGCCTTTCCGATT	CGTCGGTTTGGCCTAGGGAA	GTCTTGTGAACCTTATCACT	1670
MO-1		AGTGATCCGGTGAATTATTC	GGACCGTGGCCTTTCCGATT	CGTCGGTTTGGCCTAGGGAA	GTCTTGTGAACCTTATCACT	
Bdiv		AGTGATCCGGTGAATTATTC	GGACCGTGGCCTTTCCGATT	CGTCGGCTTGGCCTAGGGAA	GTCTTGTGAACCTTATCACT	
KY/NR		TAAAGGAAGGAGAAGTCGTA	ACAAGGTTTCCGTAGGTGAA	CCTGCAGAAGGATC (1724)		
MO-1		TAAAGGAAGGAGAAGTCGTA	ACAAGGTTTCCGTAGGTGAA	CCTGCAGAAGGATC (1724)		
Bdiv		TAAAGGAAGGAGAAGTCGTA	ACAAGGTTTCCGTAGGTGAA	CCTGCAGAAGGATC (1724)		

FIG. 3. The identical SSU rRNA gene sequences from the Kentucky human (KY) and Nantucket Island cottontail rabbit (NR) *Babesia* spp. (accession numbers AY887131, AY887132, and AY904043) depicted as one and aligned with Missouri human isolate MO-1 (accession number AY048113) and *B. divergens* (Bdiv) isolate U16370 (accession number AY046576) SSU rRNA gene sequences. Three base differences in the *B. divergens* sequence are underlined and in bold type.

FIG. 4. Transmission electron micrographs of extracellular *Babesia* sp. piroplasms (merozoite stage) cultured in cottontail rabbit erythrocytes at passage 11 (A) or human erythrocytes at passage 10 (B). LM, parasite limiting membrane; IMC, inner membrane complex; AC, apical complex; Rh, rhoptries; m, mitochondrion-like structure; N, nucleus; ER, endoplasmic reticulum. Remnants of the host erythrocyte membrane are evident in close contact to the piroplasm in panel B (RBC). Scale bars, 0.5 μ m.

cottontail rabbit serum. Substitution of domestic New Zealand White rabbit (*Oryctolagus cuniculus*) serum and RBC was attempted, but these did not support the parasite. In addition, media that successfully supported parasite growth in cottontail rabbit or human erythrocytes were unable to support the parasite in domestic-rabbit erythrocyte cultures. Although FBS has been shown to support in vitro growth of several *Babesia* spp. (15, 19, 22), as mentioned above, it did not support the cottontail rabbit parasite in this study.

Cottontail rabbit parasite cultures were established by using erythrocytes from two animals identified as positive for the presence of babesias by PCR, whereas our attempts to establish cultures from PCR negative blood were not successful (data not shown). From the primary cultures initiated using autologous erythrocytes, first passages into donor cottontail or human erythrocytes were generally successful when passaged from cultures in HL-1 medium supplemented with human serum. Early-passage parasites cultured in cottontail rabbit RBC in MEM Alpha did not readily adapt to subcultures with human RBC. However, by passage 5, the parasites were successfully introduced to human RBC, leading to the establishment of continuous cultures in human serum-supplemented medium with human erythrocytes. The morphologies of the parasites were similar whether the parasites were cultured in cottontail rabbit or human erythrocytes, as determined by light and transmission electron microscopy. This culture system provides a convenient in vitro protocol for maintaining a laboratory

source of the cottontail rabbit parasites, since both human culture components are available from commercial sources.

The life cycles of the causative agents of acute babesiosis for patients in Missouri, Kentucky, and Washington are not known (4, 9, 10). Given the molecular identity of the infecting parasite from the Kentucky case and that from Nantucket cottontail rabbits, it is likely that the vector tick is *Ixodes dentatus*, which has been reported from much of the eastern United States; the closely related *Ixodes spinipalpis* appears to replace *I. dentatus* on lagomorphs in the western United States (16). Accordingly, it is likely that babesiosis due to this parasite may occur in virtually any area of the United States. Our successful continuous laboratory propagation of this parasite will provide serological antigens, which will permit estimating its public health burden.

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