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Detection of *Babesia microti* by Polymerase Chain Reaction†

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Human babesiosis, which is caused by infection with the intraerythrocytic malarialike protozoan *Babesia microti*, has recently been diagnosed with increasing frequency in residents of New England. Diagnosis is difficult because of the small size of the parasite and the sparse parasitemia that is characteristic of most infections with this pathogen. We generated *B. microti*-specific DNA sequence information by universal primer amplification of a portion of the eukaryotic 16S-like gene; this was followed by direct DNA sequence analysis. Specific primers were synthesized on the basis of this sequence information for use in the polymerase chain reaction (PCR). The PCR-based system demonstrates a strong bias for detection of *B. microti* as opposed to *Babesia gibsoni* and does not amplify vertebrate DNA. The analytical sensitivity of the system is approximately three merozoites. Blood specimens from 12 patients with clinically diagnosed and parasitologically confirmed babesiosis from Nantucket Island, Mass., were PCR positive in a blinded test of this procedure. Thus, DNA amplification may provide an adjunct to conventional methods for the diagnosis of human babesiosis and may provide a new means of monitoring therapy or enhancing epidemiological surveillance for this emerging pathogen.

The members of the genus *Babesia* comprise nearly 100 species of tick-transmitted protozoal pathogens that infect a wide variety of vertebrate hosts (24, 36, 50). The organism was first described in 1888 by Hungarian naturalist Victor Babes (3) as the agent responsible for as many as 50,000 annual deaths among cattle in Romania during the late nineteenth century. The rodent parasite *Babesia microti* (in the United States) and the bovine pathogen *Babesia divergens* (in Europe) appear to be responsible for virtually all of the known human zoonotic cases (36, 50).

The distribution of *B. microti* in the United States parallels that of another well-known zoonotic agent, *Borrelia burgdorferi*, the causative agent of Lyme disease (6, 36, 48, 49). The white-footed mouse (*Peromyscus leucopus*) serves as the main reservoir host for *B. microti* and *B. burgdorferi*, and *Ixodes dammini* is the predominant tick vector for both pathogens. Although as many as 40% of nymphal ticks infected with the Lyme disease spirochete may be coinfected with *B. microti* (27, 38), the number of reported cases of human babesiosis is far smaller than that of Lyme disease, probably because of the tendency of *B. microti* to produce a subclinical infection (12, 15, 18, 20, 32). A 1980 study of residents of Shelter Island, N.Y., suggested that the rate of seroconversion during each transmission season was approximately 6% (17). A recent study of 1,000 *B. burgdorferi*-seropositive patients from Connecticut indicated that 10% were coinfected with *B. microti* (23). Thus, exposure to *B. microti* may be frequent; the evolution to overt clinical disease probably depends on host factors such as age, splenic function, and the competence of the individual's immune system.

The clinical diagnosis of human babesiosis is difficult, especially in the early stages; the nonspecific symptoms of malaise, anorexia, and fatigue may be easily confused with the symptoms of other diseases, such as influenza (7, 12, 43). The definitive laboratory diagnosis of babesiosis rests upon demonstration of characteristic intraerythrocytic inclusions on Giemsa-stained thin blood films (9, 16, 43). However, the relative scarcity of parasites during the early stages of disease and in many stages of disease in the normosplenic host renders this test not only insensitive but dependent on an experienced microscopist (16). Although inoculation of patient blood into hamsters serves to amplify the parasitemia to detectable levels, the animals must be monitored for up to 6 weeks postinoculation, which is impractical for diagnostic purposes (8, 16, 25). The current method of choice, an indirect immunofluorescence antibody test with antigen derived from infected hamsters, detects antibody to the organism but does not always discriminate between patients who have been exposed and those who are actively infected (11, 17, 40). A rapid and sensitive detection system based on in vitro amplification of *B. microti*-specific DNA sequences may thus facilitate a clinical diagnosis of human babesiosis.

In a previous report (13), we described the isolation of a *B. microti*-specific fragment from a gene encoding the small-subunit rRNA (ss-rDNA) and its use in discriminating *B. microti* from its morphologically indistinguishable relative *Babesia gibsoni* (the canine pathogen) (14, 33) by restriction fragment length polymorphism analysis. In this report we describe the DNA sequence analysis of this clone and its use as a genetic target for a polymerase chain reaction (PCR)-based detection system (44). The clinical utility of this system was confirmed by testing blood samples from a number of recent symptomatic cases of human babesiosis.

MATERIALS AND METHODS

Babesial strains. *B. microti* isolates were obtained from infected humans or white-footed mice (*P. leucopus*) after

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amplification and serial passage in golden Syrian hamsters as described previously (13). Isolates 1Bm (GI strain) (37), 2Bm, and 7Bm were obtained from humans infected on Nantucket Island, Mass. Isolates 3Bm and 4Bm were obtained from infected humans from Connecticut; isolates 5Bm and 6Bm were obtained from *P. leucopus* trapped near the homes of the patients in Connecticut (1, 32). *B. gibsoni* isolates were obtained from splenectomized dogs that were experimentally inoculated with blood from naturally infected dogs from southern California, as described previously (14). Isolates 1Bg, 2Bg, and 7Bg were obtained from three independent canine infections; isolates 3Bg and 4Bg used in this study were obtained after canine passage of isolate 2Bg.

DNA sequence analysis of a *B. microti*-specific DNA clone. A 589-bp *B. microti*-specific ss-rDNA fragment was produced by universal primer amplification as described previously (13, 30). A relatively large amount of input DNA (1 μg from strain GI) was used to reduce the probability of *Taq* polymerase incorporation errors (22). Two clones containing inserts in the opposite orientation were obtained by using the TA cloning vector system (Invitrogen, La Jolla, Calif.). Both clones were sequenced by using a Sequenase kit (U.S. Biochemicals Co., Cleveland, Ohio) according to the manufacturer's instructions. The sequences of the two clones were identical; however, to further reduce the probability of an erroneous sequence determination because of *Taq* polymerase incorporation errors, we reamplified the 589-bp fragment and subjected it to direct sequence analysis using a cycle sequencing kit from GIBCO/BRL (Gaithersburg, Md.). Sequence construction was facilitated by using the SEQED program (University of Wisconsin Genetics Computer Group), and sequence alignments were performed by using the GAP sequence alignment program (University of Wisconsin Genetics Computer Group) in the Mayo Foundation Research Computing Facility. Gap and length weights were assigned at 5.00 and 0.3, respectively.

DNA preparation. Genomic DNA was prepared from babesial merozoite preparations as described previously (13). Briefly, plasma and leukocytes were removed from parasitemic blood by differential centrifugation (21) and were washed in cold phosphate-buffered saline (pH 7.4). After resuspension in medium M199 (GIBCO Laboratories, Grand Island, N.Y.), merozoites were allowed to accumulate in the medium during an overnight incubation at 37°C. The erythrocytes were then pelleted by centrifugation (16,000 × *g* for 10 min at 4°C), and the resulting supernatant was removed and centrifuged again under the same conditions. The resulting pellet was recovered for DNA extraction as described previously (13, 21). Control DNA was prepared from leukocytes that were separated from uninfected human, canine, and hamster blood by differential centrifugation (400 × *g*, 20 min, 4°C) on Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, N.J.) gradients. DNA concentration and purity were determined on a Perkin-Elmer Lambda 3B spectrophotometer.

Preparation of human blood specimens. Human whole-blood specimens were collected in EDTA anticoagulant and were stored at 4°C until further analysis. For sensitivity studies, percent parasitemia in blood from an infected patient was estimated by counting 20 randomly chosen fields on a Wright-Giemsa-stained thin smear. An erythrocyte count was determined in triplicate on a cell counter (model S; Coulter, Hialeah, Fla.) in order to determine the absolute number of erythrocytes. *B. microti* parasites from a 100-μl volume of 1:10 serially diluted parasitemic blood (using uninfected human blood as diluent) were concentrated by hypotonic lysis in 10 volumes of TE (10 mM Tris [pH 7.4], 1 mM EDTA) and were then centrifuged at 16,000 × *g* in 1.5-ml microcentrifuge tubes. The pellets were washed three times in TE, taking care to remove the erythrocyte ghost layer after each wash. To the pellets were added 200 μl of K buffer (50 mM Tris [pH 8.3], 1.5 mM $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween 20, 10 μg of proteinase K [Sigma Co., St. Louis, Mo.] per ml). The pellets were dispersed by vortexing and were then incubated at 55°C for 1 h, incubated for 10 min at 95°C to inactivate the protease and denature the genomic target DNA, and then cooled immediately on ice. Processing of additional specimens from *B. microti*-infected and control patients was as described above, except that undiluted blood was used for analysis.

PCR and chemiluminescence detection. One hundred picograms of extracted genomic DNA from babesial isolates or 5 μl of the proteinase K digest was added to a 50-μl PCR containing 50 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 200 mM (each) deoxyribonucleoside triphosphate, 0.25 U of *Taq* polymerase (Perkin-Elmer Cetus), and 50 pmol of each of primers Bab1 (5′-CTTAGTATAAGCTTTTATACAGC-3′) and Bab4 (5′-ATAGGTCAGAAACTTGAATGATACA-3′). Amplification was performed in a programmable thermal cycler (Coy, Ann Arbor, Mich.) with a three-step cycling program, as follows: 1 min of denaturation at 94°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C for a total of 35 cycles. The 238-bp amplification product was visualized on an ethidium bromide-stained 2% Seakem agarose gel (FMC Bioproducts, Rockland, Maine). Amplification products were denatured in the gel and transferred onto nylon filters (Hybond NT; Amersham Corp., Arlington Heights, Ill.) by Southern blotting as described previously (34). DNA was cross-linked to the membrane by using a Stratalinker UV cross-linker set at a predetermined dosage of 1,200 J.

To generate a chemiluminescent internal probe, 1 to 10 ng of the Bab1-Bab4 amplification product was reamplified with nested primers Bab2 (5′-GTTATAGTTTATTTGATGTTC GTTT-3′) and Bab3 (5′-AAGCCATGCGATTCGCTAAT-3′) by using the same reaction conditions described above, except that 50 cycles were used in order to maximize the yield. The 154-bp internal product was then gel purified and labeled according to the manufacturer's instructions for the enhanced chemiluminescence gene detection system (Amersham). After a 1-h prehybridization in the blocking buffer provided with the kit, labeled probe (10 ng/ml) was added to the hybridization solution and the filter was incubated overnight at 37°C with gentle agitation. After hybridization, the filters were washed twice for 20 min for each wash in the enhanced chemiluminescence wash buffer containing 0.1× SSC (20× SSC is 0.3 M sodium citrate [pH 7.0] and 3 M NaCl) with 0.17 M urea and 0.01 M sodium dodecyl sulfate, and then they were washed twice for 5 min for each wash in 2× SSC. Signal generation and detection were performed in a darkroom as described by the manufacturer. Filters were exposed to Kodak X-Omat film for 15 min.

Nucleotide sequence accession number. The GenBank accession number for our *B. microti* sequence is M93660.

RESULTS

Sequence analysis of the *B. microti* ss-rDNA amplification product. Universal primer ss-rDNA amplification of partially purified *B. microti* DNA yielded a 589-bp amplification product with 514 bp of unique *B. microti* sequence information; the DNA sequence of the product is shown in Fig. 1. Sequence analysis was performed in two ways: by supercoil

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B. microti.....AGTCATATGCTTGTCTTAAAGATTAAGCC 29
P. falciparum.....AGTCATATGCTTGTCTCAAAGATTAAGCC 50

30 ATGCATGTCTTAGTATA...AGCTTTTATACAGCGAAACTGCGAATGGCT 76
51 ATGCAAGTGAAAGTATATATATATTTTATATGTAGAAACTGCGAACGGCT 100

77 CATTAAAACAGTTATAGTTTATTTGATGTTCGTTTTACATGGATAACCGT 126
101 CATTAAAACAGTTATAGTCTACTTGACATTTTTATTATAAGGATAACTAC 150

127 GGTAATTCTAGGGCTAATACATGCTCGAGGCGCGT.....TTTC 165
151 GGAAAAGCTGTAGCTAATACTTGCTTTATTATCCTTGATTTTTATCTTTG 200

166 GCGTGGCGTTTATTAGAC..TTTAACCAACCCTTCGGGTAATCGGTGATT 213
201 GATAAGTATTTGTTAGGCCTTATAAGAAAAAAGTTATTAACTTAAGGAAT 250

214 CATAATAAATTAGCGAATCGCA.....TGGCTTTGCCGGCGATGTATCA 257
251 TATAACAAAGAAGTAACACGTAATAAATTTATTTTATTTAGTGTGTATCA 300

258 TTCAAGTTTCTGACCTATCAGCTTTGGACGGTAGGGTATTGGCCTACCGG 307
301 ATCGAGTTTCTGACCTATCAGCTTTTGATGTTAGGGTATTGGCCTAACAT 350

308 GGCGACGACGGGTGACGGGGAATTGGGGTTCGATTCCGGAGAGGGAGCCT 357
351 GGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGGAGCCT 400

358 GAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCA 407
401 GAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCA 450

408 ATCCTGACACAGGGAGGTAGTGACAAGAAATAACAATACAGGGC....TT 453
451 ATTCTAAAGAAGAGAGGTAGTGACAAGAAATAACAATGCAAGGCCAATTT 500

454 AAAGTCTTGTAATTGGAATGATGGGAATCTAAACCCTTCCCAGAGTATCA 503
501 TTGGTTTTGTAATTGGAATGGTGGGAATTTAAAACCTTCCCAGAGTAACA 550

504 ATTGGAGGGCA
551 ATTGGAGGGCA
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FIG. 1. Sequence alignment of ss-rDNA fragments of *B. microti* and *P. falciparum*. A 589-bp universal ss-rDNA primer amplification reaction of genomic *B. microti* GI DNA yielded 514 bp of *B. microti*-specific sequence information (top strand). The bottom strand is the homologous sequence of *P. falciparum*. Sequence alignment was performed by using the GAP program (University of Wisconsin Genetics Computer Group). Gap and length weights were assigned at 5.00 and 0.3, respectively.

sequencing of two subclones of the amplification product and by direct cycle sequencing of the product (42). The latter approach is considered to be less prone to sequence errors because *Taq* polymerase incorporation errors, which occur at a frequency of approximately 10^{-4} to 10^{-5} nucleotides (22), are averaged out when the entire population of amplified products is sequenced. Both methods, however, yielded identical sequences, presumably because the amplification reactions were initiated with relatively large amounts of input DNA so that a smaller number of cycles (resulting in fewer opportunities for error) was needed to reach saturation.

Sequence alignment of the *B. microti*-specific sequence to known ss-rDNA sequences from other intraerythrocytic protozoal pathogens yielded a surprisingly low level of homology. Alignment with the corresponding region of *Plasmodium falciparum* demonstrated only 75% homology (Fig. 1) (29). Poor homology was also observed with other members of the genus *Plasmodium*, *P. vivax* (75%) (52) and *P. lophurae* (76%) (53) (data not shown). An expected low level of homology was also observed with the human and rat ss-rDNA sequences (75 and 74%, respectively) (10, 28); divergence from the cognate human sequence is important for the development of specific diagnostic reagents for use in human blood. The lack of homology with the rodent sequence is important because small amounts of rodent host cell DNA regularly contaminate preparations of *B. microti* merozoites (13); universal primer amplification could result in a mixed population of molecules of rodent and babesial origin. Sequence analysis thus confirmed the identity of the *B. microti*-specific product.

PCR amplification of *B. microti* DNA. A specific diagnostic primer pair was developed for PCR detection of *B. microti* by comparing the sequence of the *Babesia* ss-rDNA fragment with known ss-rDNA sequences from related and unrelated organisms. Amplification of *B. microti* DNA by using primers Bab1 and Bab4 would be expected to yield a 238-bp amplification product (Fig. 2); although Bab4 lies in a region of high homology between *B. microti* and another member of the genus, *Babesia bigemina* (41) (data not shown), Bab1 overlies a region with significant divergence toward its 3′ end and would thus be expected to provide specificity. All of the seven isolates of *B. microti* from humans and rodents produced an amplification product of equal intensity that migrated at approximately 240 bp (Fig. 3, lanes 2 to 8); with isopsoralen sterilization, the electrophoretic mobility of the product was reduced so that it migrated at approximately 330 bp (data not shown). In contrast, four isolates of the closely related canine pathogen *B. gibsoni* failed to demonstrate an amplification product on an ethid-

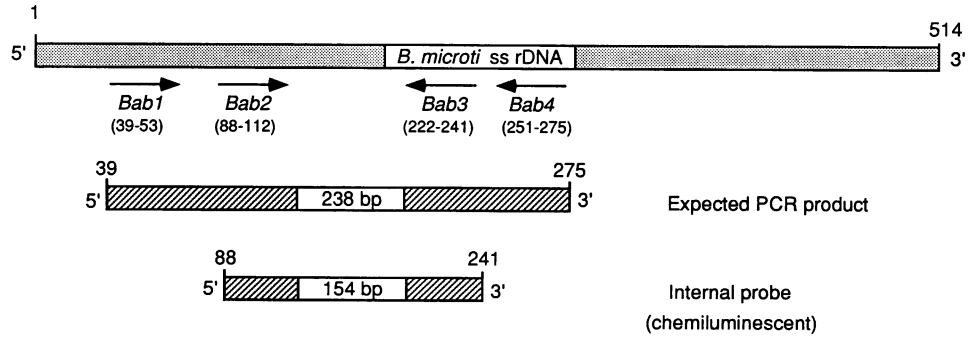

FIG. 2. Position of *B. microti*-specific primers and the chemiluminescent probe. Primers Bab1 and Bab4 were used in the PCR to produce a 238-bp amplification product; the binding site for Bab1 lies in a *B. microti*-specific region, whereas that of Bab4 lies in a region of high homology between *B. microti* and *B. bigemina* (41). Primers Bab2 and Bab3 were used in a PCR to produce a 154-bp internal fragment for use as a chemiluminescent probe. The numerical positions delineating the amplification products are listed above the products.

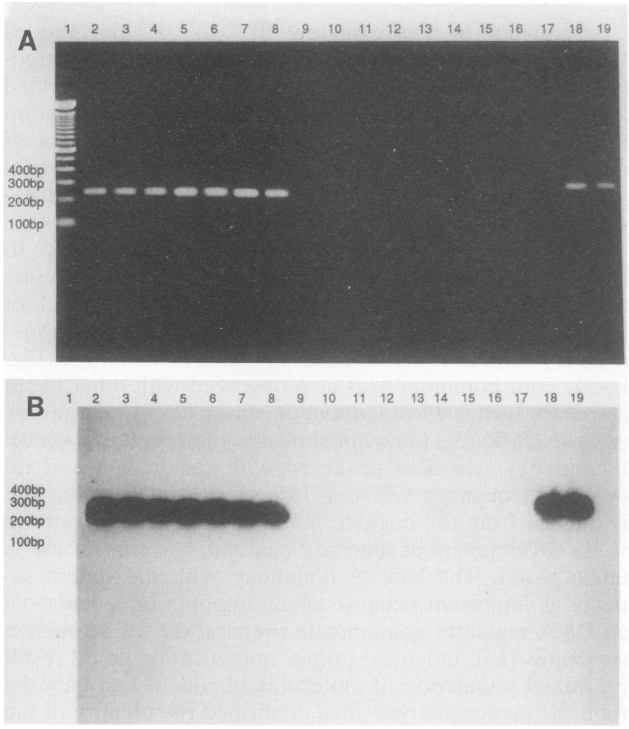

FIG. 3. Detection of a *B. microti*-specific DNA target in preparations of babesial genomic DNA and naturally infected human blood. (A) Primers Babl and Bab4 were used in a PCR to detect a *B. microti*-specific target within genomic DNA prepared from seven isolates of *B. microti* (lanes 2 to 8) and four isolates of *B. gibsoni* (lanes 11 to 14). Lanes 9, 10, 15, and 16, no-target-DNA controls; lanes 18 and 19, identical reactions performed on lysates of 100 μl of human blood from two independent cases of human babesiosis; lane 17, uninfected human blood control; lane 1, a 100-bp ladder (the sizes of the relevant markers are shown on the left). (B) Hybridization of the reaction products shown in panel A to a 158-bp chemiluminescent internal probe.

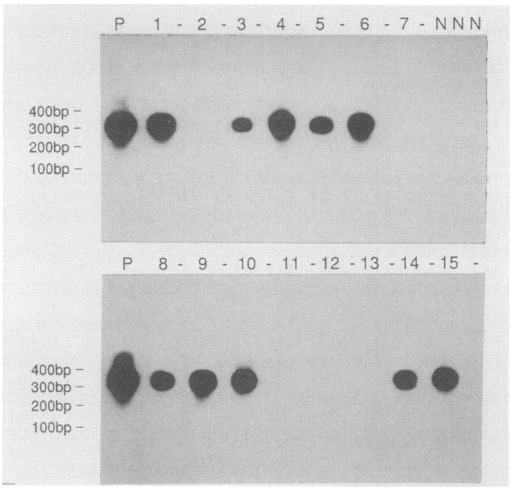

FIG. 4. Detection of *B. microti* in blood from patients with babesiosis. Blood from 10 seropositive patients with babesiosis (lanes 1, 3 to 6, 8 to 10, 14, and 15) who presented to Cottage Hospital (Nantucket, Mass.) in 1990 and 1991 was subjected to the lysis-centrifugation procedure, and DNA was amplified by using primers Babl and Bab4. Blood from five uninfected patients (lanes 2, 7, and 11 to 13) and three random specimens (lanes N) were included as controls. Negative-reagent controls (no-target-DNA controls) were interspersed between all patient specimens (lanes labeled with a minus sign). Reactions were isopsoralen cross-linked, electrophoresed, blotted, and probed as described in the text. The electrophoretic mobility of the *B. microti*-specific product was retarded by the presence of isopsoralen cross-links, so that it migrated at approximately 330 to 350 bp. Lanes P, positive control reactions.

ium-stained gel. However, when it was hybridized to a chemiluminescent internal probe, the filter generated from the gel showed a weak hybridization signal from one of the *B. gibsoni* isolates (Fig. 3, lane 14). None of the no-target-DNA (Fig. 3, lanes 9, 10, 15, and 16) or genomic DNA (Fig. 3, lane 17) controls showed a demonstrable signal. One explanation for the low-level signal seen for one of the *B. gibsoni* isolates is limited cross-species reactivity of our reagents; however, it is also possible that small amounts of *B. microti* genomic DNA were introduced into the *B. gibsoni* DNA preparation during sample handling or processing.

Direct detection of *B. microti* in human blood. We next analyzed blood specimens from cases of human babesiosis. Blood specimens were obtained from 12 cases of human babesiosis presenting to the Nantucket Cottage Hospital in 1990 and 1991. All patients presented with the following typical features of acute babesiosis: a few days to weeks of nausea, malaise, extreme fatigue, and in some cases, hematuria. Blood smears prepared at the time of presentation demonstrated intraerythrocytic inclusions consistent with *B. microti*, and all infections were serologically confirmed by indirect fluorescent antibody reactivity greater than 1:128 as described previously (23). Aliquots of 100 μl of blood from two of these patients were subjected to a rapid hypotonic lysis-centrifugation procedure to concentrate the merozoites; aliquots of protease- and detergent-treated lysates from the concentrates were directly amplified with primers Babl and Bab4. The results shown in Fig. 3A and B were obtained after amplification of digested blood lysates containing 0.5% (lane 18) and 1.2% (lane 19) erythrocytic inclusions. Consistent with the relatively large number of parasites expected to be present in these specimens (a 100-μl aliquot of 1% parasitemic blood would be expected to contain approximately 10^6 merozoites), a strong amplification signal was observed on an ethidium-stained gel.

Southern blot analysis of amplification reactions performed on the remaining 10 specimens and on additional controls is shown in Fig. 4. All *B. microti*-positive specimens (as determined by microscopic analysis or serology) were positive by PCR (Fig. 4, lanes 1, 3 to 6, 8 to 10, 14, and 15). All of the control specimens, consisting of three specimens collected at random from the Mayo Clinic hematology laboratory and five smear-negative, antibody-negative specimens from patients living in an endemic area, were negative. A sample from the patient represented in lane 4 of Fig. 4, which was obtained 1 week after the initiation of therapy, was PCR positive, even though the smear was negative. No-target-DNA controls were interspersed with patient samples to control for amplicon carryover (Fig. 4, lanes labeled with a minus sign).

In order to determine the sensitivity of PCR detection, we performed 1:10 serial dilutions on one of these specimens using uninfected human blood as the diluent. The number of organisms in each reaction was determined by estimating the percentage of infected erythrocytes within 20 randomly chosen fields of a Wright-Giemsa-stained thin smear along with quantitation of the number of erythrocytes. The undiluted specimen contained approximately 300,000 merozoites

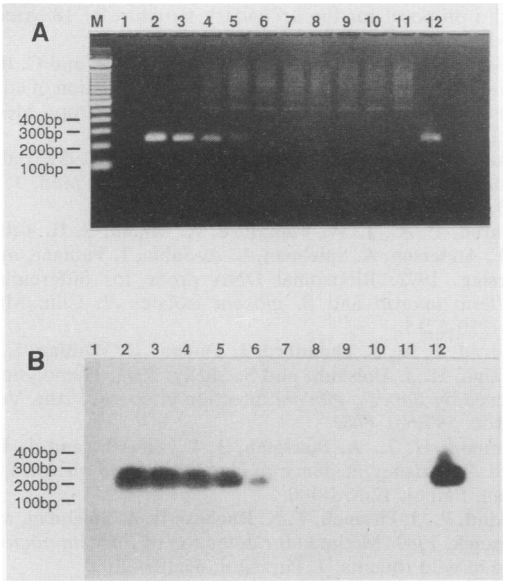

FIG. 5. Sensitivity of PCR detection of *B. microti* in human blood lysates. Infected blood from one of the human cases was diluted in a 1:10 dilution series by using uninfected human blood as diluent. Aliquots of 100 μl were processed by hypotonic lysis-centrifugation; 5 of 200 μl of the resuspended pellet was amplified in a 50-μl reaction. (A) Ethidium-stained agarose gel. Lanes M, 100 bp ladder. Reaction products from the following dilution series of *B. microti* merozoites were tested: 3×10^4 (lanes 2), 3×10^3 (lanes 3), 3×10^2 (lanes 4), 3×10^1 (lanes 5), 3×10^0 (lanes 6), 3×10^{-1} (lanes 7), 3×10^{-2} (lanes 8), and 3×10^{-3} (lanes 9). Lanes 10 and 11, normal human blood controls; lanes 12, *B. microti* genomic DNA GI positive control. (B) Hybridization of the reaction products shown in panel A to the chemiluminescent internal probe.

per 100-μl aliquot. Because only 1/50th of the processed specimen was used for each reaction, the single-organism level was reached at the sixth dilution. The results of PCR amplification of the dilution series are shown in Fig. 5. A *B. microti*-specific amplification product could be seen on an ethidium-stained gel from the fourth dilution; the signal corresponded to approximately 30 organisms (Fig. 5A, lane 5). Hybridization to the chemiluminescent internal probe improved the sensitivity of the PCR by an order of magnitude (Fig. 5B, lane 6), resulting in detection of approximately three merozoites in a 50-μl reaction. Similar results were observed with specimens taken from other patients, suggesting that the sensitivity of the system is not isolate specific (data not shown).

DISCUSSION

The emergence of *B. microti* as an important human pathogen in the United States was recently documented with the outbreak of Nantucket fever among residents of the terminal moraine islands of the coast of New England (19, 39, 43, 47). In contrast to earlier cases (43), most of the victims of this outbreak were normosplenic, suggesting that significant disease risk now exists for the general population in those areas. Recently, seven human infections were diagnosed in residents of coastal Connecticut (1), indicating that enzootic areas have now become established on the U.S. mainland. Because *B. microti* shares a vertebrate host reservoir and tick vector with *B. burgdorferi*, it might be expected that the caseload for human babesiosis will parallel the rise in the number of cases of Lyme disease in endemic areas (6, 15, 47).

The clinical course of human babesiosis often seen in asplenic hosts or elderly individuals (fever, drenching sweats, hemolytic anemia, and hemoglobinuria) shares many features with malaria (7, 43). Likewise, the many intraerythrocytic merozoite inclusions seen on a stained blood film may be easily mistaken for early ring-form trophozoites of *P. falciparum*. However, ultrastructural studies place the piroplasms and the malarial parasites within separate taxonomic orders (24). Thus, we expected substantial phylogenetic divergence between the two pathogens on the basis of a comparison of a portion of the gene encoding the nuclear ss-rRNA subunit. Whereas the sequence conservation of this portion of the gene was quite high (ca. 75% homology between vertebrate and *B. microti* genes), the homology to *Plasmodium* sp. was only slightly better at ca. 77% identity. The other known ss-rDNA sequence of the genus *Babesia* is that derived from *B. bigemina*, a bovine parasite endemic to Central America (24, 31, 41). A higher level of sequence homology with *B. microti* (88%; data not shown) was observed when the *B. microti* sequence was aligned with the cognate sequence from *B. bigemina*. However, this level of homology is still not as high as might be expected for members of the same genus. (The most distant members of the genus *Plasmodium*, for instance, demonstrate ca. 90% homology for this same portion of the ss-rRNA gene.) The babesias have been classified into two morphologically distinct groups: the "small babesias," which include *B. microti*, *B. gibsoni*, and *B. equi*, and the large babesias, which include *B. bigemina*, *B. canis*, and *B. divergens*. Distinguishing the two groups may be important on pathophysiological grounds; the small babesias appear to lack transovarial transmission (51) and may have an exoerythrocytic stage (31, 45). The substantial divergence of *B. bigemina* from *B. microti* may be another indicator of the underlying differences between these pathogens. Comparison of ss-rDNA sequences is one of the most powerful methods available for establishing evolutionary relationships (30, 35, 54). However, conclusive evidence of the relationship of *B. microti* to members of the same genus and to other intraerythrocytic pathogens must await determination of the complete *Babesia* ss-rDNA sequence.

The microscopy-based diagnosis of babesiosis presents a variety of difficulties. Few laboratories are staffed with personnel with enough parasitological expertise to carefully prepare and evaluate blood smears. The small size of the piroplasm does not allow for confident recognition of the organism within thick blood smears. Thus, the thin blood smear has mainly been used for the detection of *B. microti*. However, the threshold of detection for malarial parasites by this technique is 100/μl (9), and many babesial infections may be missed. Indeed, thin blood smears detected only about 60% of infections in wild-caught *P. leucopus* when compared with the amount detected by hamster inoculation (16). Although subinoculation into hamsters may detect parasitemias of 300 parasites per ml, such tests require several weeks for completion. This laborious and expensive technique may thus serve only to confirm the identity of the parasite. Radiolabeled DNA probes have been used with some success for the diagnosis of malarial infections, but the threshold of detection appears to be about 25 parasites per μl under the most refined circumstances (4). Such a threshold, in the case of babesiosis, is less sensitive than hamster subinoculation. We therefore preferred to proceed directly to a DNA amplification-based method.

The dearth of cardinal diagnostic features of human babesiosis in many patients underscores the need for a sensitive diagnostic test. Flulike symptoms during the summer months in an endemic area may be due to a variety of causes and may, in fact, be confused with early Lyme disease (49). We recently described a PCR-based direct detection method for the Lyme disease spirochete and applied it to the detection of *B. burgdorferi* this pathogen in field-derived *I. dammini*, archived museum specimens, and animal and human tissues (2, 5, 26, 34). As for the study of Lyme disease, the addition of a direct detection method for *B. microti* to our diagnostic repertoire will be useful for the study of the pathogenesis of the disease and will provide a practical means of monitoring the course of pharmacologic therapy. Direct detection of *B. microti* in units of blood collected from donors in endemic areas may help to determine the risk of transfusion-acquired babesiosis, seven cases of which are now documented (32, 40, 46). It may also be possible to study synergistic pathogenetic effects of infection with multiple tick-transmitted pathogens by this approach (6, 27, 38). In summary, PCR provides us with a sensitive diagnostic tool for the detection of *B. microti* and will add to our understanding of the true prevalence of babesiosis and its pathogenetic course in humans.

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