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Comparison of PCR with Blood Smear and Inoculation of Small Animals for Diagnosis of *Babesia microti* Parasitemia

PETER J. KRAUSE,[1]* SAM TELFORD III,[2] ANDREW SPIELMAN,[2] RAYMOND RYAN,[3] JENIFER MAGERA,[4] T. V. RAJAN,[5] DIANE CHRISTIANSON,[1] TOD V. ALBERGHINI,[6] LAURINE BOW,[6] AND DAVID PERSING[4]

Departments of Pediatrics,[1] Laboratory Medicine,[3] Medicine,[6] and Pathology,[5] University of Connecticut School of Medicine, Farmington, Connecticut; Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts[2]; and Department of Laboratory Medicine and Pathology, Mayo Clinic, Rochester, Minnesota[4]

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The specific diagnosis of babesiosis, which is caused by the piroplasm *Babesia microti*, is made by microscopic identification of the organism in Giemsa-stained thin blood smears, detection of babesial antibody in acute- and convalescent-phase sera, or identification of the organism following the injection of patient blood into laboratory animals. Although rapid diagnosis can be made with thin blood smears, parasites are often not visualized early in the course of infection. PCR is a new, rapid diagnostic technique for the detection of *Babesia* spp. that has not yet been systematically evaluated. We conducted a blinded study of the sensitivity, specificity, and reproducibility of the PCR-based test with patients with babesiosis and a group of asymptomatic subjects residing in a region in southern New England where babesiosis is enzootic. Among 19 patients with recent babesial illness, we found that PCR was as sensitive and specific as the use of Giemsa-stained blood smears and inoculation of hamsters. Among asymptomatic subjects, the PCR result was positive for 3 persons with recent babesial infection and was negative for 41 persons without previous babesial infection. We conclude that the *B. microti* PCR procedure is sufficiently sensitive, specific, and reproducible for use in the diagnosis of acute babesiosis.

Babesiosis is an emergent zoonosis caused by an intraerythrocytic piroplasm with many clinical features that are similar to those of malaria (4, 15). Since the initial report of human babesiosis in 1957, the epidemiology of the disease has changed from a few isolated cases to the identification of areas in the northeastern and northern midwestern United States where the disease is endemic, and cases of the disease have been reported over a wide geographic range in Europe and North America (1, 3, 8, 10, 12, 14–16). The majority of patients with babesiosis incur a mild illness or are asymptomatic, while some develop severe illness that may result in death.

The specific diagnosis of babesiosis initially is made by microscopic identification of the organism by Giemsa or Wright staining of thick or thin blood smears (4). Multiple thick and thin blood smears should be examined, because usually less than 1% of erythrocytes are parasitized in the first week of the illness, when most people seek medical attention. Since initial blood smears may be negative, physicians have had to rely on less rapid methods of diagnosis, including the detection of antibabesial antibody and inoculation of small animals (2, 5, 7). The recent development of a PCR for the detection of *Babesia microti* has made available an additional rapid diagnostic method (11, 13). This method has not yet been systematically evaluated, however. In the present study, we compared the sensitivity and specificity of a *B. microti* PCR technique with that of blood smear and inoculation of hamsters for the detection of babesiosis in human patients.

* Corresponding author. Mailing address: Department of Pediatrics, University of Connecticut School of Medicine, Farmington, CT 06030. Phone: (860) 545-9494. Fax: (860) 545-9590.

MATERIALS AND METHODS

Study population. Blood samples were obtained from 448 Block Island, R.I., residents (233 females and 215 males; mean age, 45 years) who participated in a yearly serosurvey for the detection of *B. microti* antibody that included a questionnaire regarding health status and deer tick exposure in the previous year. Blood was also obtained from 24 Block Island, southeastern Connecticut, and Nantucket, Mass., residents who were evaluated for possible babesial infection from 1991 to 1993. For each clinically ill subject, a history was obtained and a physical examination and laboratory tests were performed, including Giemsa-stained blood smears for babesia, indirect immunofluorescence assay (IFA) for anti-*B. microti* antibody, enzyme linked immunosorbent assay (ELISA) for anti-*Borrelia burgdorferi* antibody, PCR assay for *B. microti*, and hamster inoculation assay for *B. microti*. Informed consent was obtained from each study participant. The study was carried out in accordance with the guidelines of the Human Subjects Committee of the Harvard School of Public Health. A subject was considered to have acute babesiosis if (i) a fourfold rise in anti-*B. microti* antibody was detected in sera obtained from serosurvey subjects before and after a summer babesial transmission season or (ii) consistent symptoms and supportive laboratory data were observed in patients with babesiosis-like illness. Consistent symptoms included fever and at least one of the following: chills, fatigue, myalgias, or sweats. Laboratory evidence of babesiosis included the detection of piroplasms either on a smear of peripheral blood or indirectly through the hamster inoculation assay or observation of a fourfold rise in antipiroplasm immunoglobulin G (IgG) antibody in acute- and convalescent-phase sera.

Preparation of blood smears. Piroplasms were diagnosed microscopically in Giemsa-stained films of EDTA-anticoagulated blood (4). At least 100 fields (×400 magnification) were examined before the sample was declared free of piroplasms. Objects suggestive of piroplasms were further scrutinized at ×1,000 magnification.

IFA for antibabesial antibody. Babesial infection was diagnosed serologically by an IFA as described previously (5, 7). Test sera were diluted 1:32 in phosphate-buffered saline (PBS), and the titers of all serum samples were determined to their endpoint. The secondary antibody was fluorescein isothiocyanate-labeled goat anti-human IgG or IgM (Kirkegaard & Perry, Gaithersburg, Md.) diluted in PBS and Evans Blue (final concentration, 0.0005%). Slides were examined at ×630 magnification under epifluorescence. For comparison, each series of tests included serum from a subject with babesiosis (a positive control), serum from a healthy adult (a negative control), and PBS. A positive specimen was defined as one reacting at a dilution of 1:32 or greater (5, 7).

ELISA for antispirochetal antibody. Serological evidence of exposure to the Lyme disease spirochete was detected by ELISA (8, 9). A standardized whole-cell suspension containing 10^6 spirochetes (strain 2591; Connecticut Agricultural

Experiment Station, New Haven) per ml was used to coat alternate wells of flat-bottom microdilution plates. In the intervening wells, 50 μl of PBS was added to test for nonspecific binding. The plates were blocked with PBS (200 μl) containing 5% horse serum and 0.01% dextran sulfate and were washed with PBS–Tween 20. Subject sera (diluted 1:160 and 1:320) were added to matching wells, and the plates were incubated for 1 h. Following incubation with peroxidase-labeled anti-human IgG or IgM, the plates were washed again and the reactions were visualized with equal volumes of 2′,2′-azino-di-(3-ethylbenzthiazoline-sulfonate) and hydrogen peroxide (Kirkegaard & Perry) as the substrate. The optical density at 414 nm of the positive control minus the optical density of the nonspecific-binding well equaled 1.0 for IgG or 0.5 for IgM. A sample was considered reactive if the net absorbance (optical density of the antigen well minus the optical density of the nonspecific-binding well) was three or more standard deviations greater than the mean absorbance of the PBS-containing comparison wells. A reactive serum sample was defined as one reacting at a dilution equal to or greater than 1:320.

PCR assay for piroplasm DNA. One of the coinvestigators (J.M.), who was unaware of the clinical status of the study subjects, carried out the *B. microti* PCR test using a modification of a previously described assay (11, 13). In brief, whole blood was collected in EDTA anticoagulant. Total cell lysis and concentration of a 500-μl aliquot of whole blood were carried out in a hypotonic lysis buffer containing nonionic detergent (Isoton II [Coulter Diagnostics, Hialeah, Fla.]) supplemented with Triton X-100 as previously described. The blood was added to a 2-ml nonsiliconized microcentrifuge tube containing 800 μl of Isoton II. After the addition of 150 μl of 10% Triton X-100, the mixture was vortexed for 3 s and then centrifuged at maximum speed (>12,000 × *g*) in a microcentrifuge tube for 10 min. The supernatant was removed and discarded, leaving a small cellular pellet (11). Nucleic acid extraction was carried out by a modified IsoQuick method (Microprobe Corporation, Bothell, Wash.). The extracted DNA in Tris EDTA (5 μl) was added to a PCR mixture (50 μl) containing 50 pmol (each) of the oligonucleotide primers Bab 1 and Bab 4, 50 μg of isopsoralen compound 10 (for post-PCR amplicon inactivation; HRI Inc., Concord, Calif.) per ml, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.001% gelatin, 0.001% bovine serum albumin, 200 μM (each) deoxyribonucleoside triphosphates, 0.5% Tween 20, 10% (vol/vol) glycerol, and 0.25 U of Ampli-*Taq* polymerase (11). PCR was performed by 5 min of denaturation at 94°C and then 50 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 58°C, and 5 min of extension at 72°C. The reaction products were incubated at 4°C in an HRI-100 transilluminator (wavelength, 300 to 400 nm) for 15 min to inactivate the amplicons. An aliquot of the reaction product (16 μl) was mixed with loading buffer, the mixture was added to the wells of a 2% SeaKem agarose gel in TBE (Tris-borate-EDTA) buffer, and the components were electrophoretically separated. The 238-bp amplification product was stained with ethidium bromide, and a photograph was taken. The gel was denatured and neutralized, and the reaction products were transferred to a nylon membrane by Southern blotting. The reaction products were coupled to nylon membranes with a UV cross-linker set at a dose of 1.2×10^5 μJ. The amplified products were detected with an enhanced chemiluminescence gene detection system (Amersham) as described previously (11).

The controls used to prevent false-positive PCRs included strict physical separation of pre- and postamplification steps within a specially designed PCR facility and incorporation of photochemical inactivation of the PCR products, as follows: all amplification reactions contained 50 mg of isopsoralen compound 10 (HRI Inc.) per ml in the master mixture. After each amplification the microtubes were exposed to UV light to promote covalent cross-linking of the isopsoralen, thereby preventing false positivity associated with PCR product contamination. The products of amplification were detected by gel electrophoresis of 20% of the reaction volume in a 3% agarose gel containing ethidium bromide. The amplification products were transferred to a nylon membrane which was then hybridized with a chemiluminescence-labeled internal DNA probe and were detected by autoradiography. The efficiency of inactivation of the amplified DNA by isopsoralen was shown by an experiment with two sets of 10-fold serial dilutions of amplified template up to a 10^{-15} dilution. One reaction mixture contained isopsoralen and was not treated with UV light, whereas the other reaction mixture containing isopsoralen was treated with UV light. Amplification was noted in the untreated amplicon dilution set even when the target amplicons were as dilute as 10^{-12}, whereas no amplification was noted in the UV light-treated target beyond a 10^{-1} dilution. One negative control was included for every five specimens, and the samples were processed identically throughout the assay. All samples were processed in a blinded manner with regard to patient results or knowledge of a history of previous positive PCR status. None of the negative controls was falsely positive over the 3-year period of the study.

Inoculation of hamsters. Inoculation of hamsters was performed by a standard method (2). Blood was obtained from the patient (approximately 2 to 3 ml), placed in a glass tube with EDTA anticoagulant, and stored at 4°C for no more than 24 h before inoculation of hamsters. Blood was allowed to come to room temperature and 0.1 or 0.5 ml was injected intraperitoneally into 6-month-old female Syrian hamsters. The presence of *B. microti* in the hamsters was determined over 1 month by weekly examination of Giemsa-stained thin blood smears. The smears were obtained via a tail snip performed under light anesthesia.

Data analysis. Fisher's exact test with two-tailed comparisons was used to evaluate differences in PCR, blood smear, and hamster inoculation assays in detecting parasitemia. A probability of error of less than 0.05 was accepted as evidence of statistical significance.

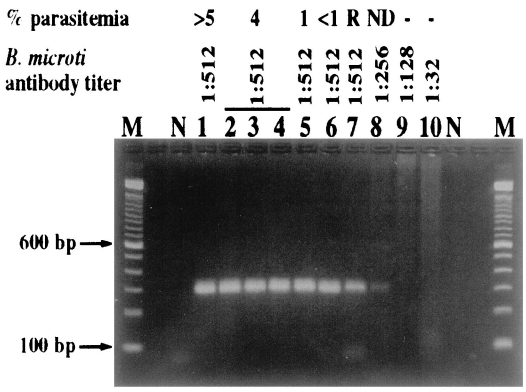

FIG. 1. PCR determination of *B. microti* DNA in serial whole-blood specimens from a patient with acute babesiosis. *B. microti* PCR tests were performed on 6 July 1992 (lane 1), 7 July 1992 (lanes 2 to 4), 8 July 1992 (lane 5), 9 July 1992 (lane 6), 10 July 1992 (lane 7), 9 September 1992 (lane 8), 6 June 1993 (lane 9), and 2 August 1993 (lane 10). *B. microti* DNA was present from 6 July through 9 September 1992 but was undetectable on 6 June and 2 August 1993. Lane M, molecular size marker; lane N, negative control. *B. microti* IFA antibody titers and percent parasitemia correspond to the dates of the PCR tests. The amount of parasitemia is expressed as the percentage of erythrocytes infected (R, rare; ND, not done).

RESULTS

First, we determined the sensitivity and specificity of the PCR assay for the detection of *B. microti* DNA in the serosurvey participants. Of 448 participants, 10 were seropositive for the piroplasm and 47 were positive for the Lyme disease spirochete (*B. burgdorferi*). Six of the 10 subjects seropositive for babesia were also seropositive for the Lyme disease spirochete. Of the 10 babesia-seropositive subjects, 3 were found to have had babesial infection within the previous 3 months on the basis of clinical and serologic evidence, including a fourfold rise in the *B. microti* IFA antibody titer. The remaining seven patients were found to have had babesial infection more than 3 months before the initial blood sample was obtained on the basis of clinical history and the presence of antibabesial IgG antibody but the absence of antibabesial IgM antibody. The *B. microti* PCR was positive for all three subjects with recent babesial infection but for only one of the seven subjects whose acute babesial infection occurred in the more remote past (Fig. 1). The *B. microti* PCR was negative for 41 subjects who had antibody to the spirochete but who lacked antibody to the piroplasm. These people served as negative control subjects because they had previous exposure to the deer tick with no evidence of babesial transmission and had reported no illness within the previous year on the serosurvey questionnaire. These data suggest that PCR is a reliable technique for the detection of acute *B. microti* infection, although patients with serologic evidence of past exposure to *B. microti* are not necessarily PCR positive.

We next compared the sensitivity and specificity of the *B. microti* PCR with those of Giemsa-stained blood smear and hamster inoculation assays for 24 patients who had had a malaria-like or flu-like illness in the spring or summer. PCR, blood smear, and hamster inoculation assays were performed within a month of the onset of illness and before antibabesial therapy. Nineteen such patients had evidence of acute babesial infection on the basis of clinical data and a fourfold rise in the

TABLE 1. Comparison of *B. microti* PCR with thin blood smear and hamster inoculation for 19 patients with acute babesiosis and 5 subjects without babesiosis[a]

Diagnostic assay	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
Thin blood smear	84	100	100	62
Hamster inoculation	74	100	100	50
PCR	95	100	100	83

[a] Of 24 patients with symptoms consistent with acute babesiosis, 19 were confirmed to have babesiosis by a fourfold rise in anti-*B. microti* antibody titer and 5 were not. The latter five patients did not meet the case definition for babesiosis and served as negative controls. PCR was more sensitive than the hamster inoculation assay for the detection of *B. microti* infection ($P < 0.05$). For 21 patients, 0.5 ml of patient blood was inoculated into the hamster peritoneal cavity, while for 3 patients (both PCR positive), 0.1 ml was inoculated. There were no significant differences in specificity or positive and negative predictive values between the PCR, blood smear, and hamster inoculation assays for the detection of *B. microti* infection in our study sample.

B. microti IFA antibody titer. These patients experienced an acute febrile illness with associated clinical and laboratory findings that met our case definition of babesial infection. Five patients lacked evidence of an acute babesial infection. One of the latter patients experienced a flu-like illness and a low concentration of babesial antibody that was consistent with infection in the past (*B. microti* IFA titer of 1:64 and no fourfold rise in antibody titer in acute- and convalescent-phase sera). Positive confirmatory test results for patients with acute babesiosis were found more frequently by PCR (18 of 19 patients) than Giemsa staining of blood smears (16 of 19 patients) or the hamster inoculation assay (14 of 19 patients; $P < 0.075$). None of these tests was positive for five patients who did not have recent babesial infection. PCR was more sensitive than (although the P value was < 0.075) and as specific as hamster inoculation and as sensitive and specific as blood smear for the detection of *B. microti* infection (Table 1).

DISCUSSION

The use of PCR for the detection of *Babesia* spp. is a new diagnostic technique that has not previously been compared with standard methods for the diagnosis of babesiosis. New methods for the diagnosis of babesiosis are needed because of the inherent limitations of traditional methods. Parasites are often missed on blood smear examination when patients with babesiosis first seek medical care, because usually less than 1% of erythrocytes are parasitized early in the course of the illness (4, 17). Antibody testing is useful for the confirmation of babesiosis, but it also may yield a negative result in the early phase of illness (5, 7). In cases in which the presence of *Babesia* spp. is suspected but not demonstrated by blood smears or antibody studies, blood from the patient can be injected by the intravenous or intraperitoneal route into small laboratory animals such as hamsters or gerbils. If present in the patient, *B. microti* usually does not appear in the blood of the test animal until 2 to 4 weeks after inoculation (2).

We found PCR to be more sensitive than (although the P value was < 0.075) and as specific as hamster inoculation and as sensitive and specific as blood smear for the detection of *B. microti*. On the basis of our findings, PCR should supplant the use of inoculation of hamsters, which is expensive and time-consuming and which requires special technical and laboratory capabilities. Giemsa-stained blood smear assays remain a reasonably sensitive, specific, and inexpensive technique for the diagnosis of acute *B. microti* infection. PCR should be used when acute babesiosis is considered and blood smear results are equivocal or negative.

B. microti PCR may be used to monitor for the presence of parasitemia in patients in the convalescent stage of babesial illness (6). The absence of detectable *B. microti* DNA in patients with serological evidence of past exposure does not necessarily indicate that such patients are free of infection, however. In naturally infected animals and experimental models, chronic infection is the rule rather than the exception. Failure to detect the parasite in a single 0.5-ml blood specimen may be due to inadequate sampling, the sporadic nature of the chronic parasitemia, or sequestration of the parasite within immunologically privileged sites such as the central nervous system or bone marrow. Increased sensitivity for the detection of the chronic carrier state, if it exists in humans, might be facilitated by analysis of larger volumes of blood or by testing for target DNA or RNA molecules that are present in multiple copies.

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