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Diagnosis of Babesiosis Using an Immunoblot Serologic Test

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Although the current indirect immunofluorescent assay (IFA) diagnostic antibody test for human babesiosis is sensitive and specific, an immunoblot antibody test may be easier to standardize and to perform. Our objective, therefore, was to determine the efficacy of and develop interpretive criteria for an immunoblot antibody test for diagnosing acute human babesiosis using a *Babesia microti* whole-cell lysate as the antigen. We compared the reactivity of sera to a *B. microti* immunoblot assay in 24 human subjects experiencing symptoms and expressing laboratory evidence of babesiosis, 28 subjects who experienced Lyme disease, 12 subjects who experienced human granulocytic ehrlichiosis, and 51 subjects who reported no history of any of these diseases and whose sera did not react against *B. microti* antigen in an IFA test. Immunoblot strips were impregnated with proteins derived from the GI strain of *B. microti* that had been electrophoresed in an acrylamide sodium dodecyl sulfate gel, followed by electroblotting onto nitrocellulose membranes. The sera of all subjects who experienced babesiosis reacted against the *B. microti* antigen in the IFA and against at least one of nine immunoblot protein bands specific to *B. microti*. In contrast, none of the sera from people who appeared not to have experienced this infection reacted against the *B. microti* antigen in the IFA (compared to 4% in the immunoblot assay). When two reactive bands were considered as definitive, immunoblot test sensitivity was 96%, while specificity was 99% and predictive positivity and predictive negativity were 96 and 99%, respectively. Our *B. microti* immunoblot procedure shows promise as a sensitive, specific, and reproducible assay for routine clinical diagnosis of acute babesiosis.

Human babesiosis due to *Babesia microti* is an emerging malaria-like infection that may be life-threatening and is endemic in parts of the northeastern and north central United States (5–7, 11, 14, 16, 17). Isolated episodes of human disease due to related pathogens have been noted elsewhere in North America, Europe, and Asia (3, 4, 12, 15). Prompt and accurate diagnosis is essential to effective case management because the condition tends to be so local and potentially so severe. Although conclusive diagnosis of this disease generally depends upon microscopic examination of thin blood smears, the pathogenic piroplasms frequently are overlooked because parasitemia tends to be sparse, often infecting fewer than 1% of erythrocytes early in the course of the illness. Serologic testing, therefore, provides useful supplementary evidence of infection, because a robust antibody response characterizes human babesial infection, even at the time when parasitemia first becomes detectable (6, 8, 9). The present serological diagnostic standard relies on a time-consuming and exacting indirect immunofluorescence antibody assay (IFA) using antigen derived from infected hamsters (2, 8, 9). To facilitate routine diagnosis of human babesiosis, therefore, we evaluated the test characteristics of an immunoblot diagnostic assay for human babesiosis that can be conducted by generalist personnel and that uses reagents that can be mass produced. In particular, we compared the immunoblot reactivities against the *B. microti* antigen of sera sampled from babesial patients with those of patients suffering from Lyme disease and from human granulocytic ehrlichiosis (HGE). Sera from asymptomatic subjects were included as controls. In addition, the results of these immunoblot tests were compared to those based on conventional IFA.

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MATERIALS AND METHODS

Study population. Blood was sampled from 24 adult New England residents 1 to 18 months after they developed clinical evidence of babesiosis. Acute and convalescent sera were available and were tested for 10 of the 24 subjects for a total of 34 serum samples tested. The presence of piroplasms was confirmed microscopically in thin smears of samples from 9 of these 24 subjects, and *B. microti* DNA was amplified from the samples from the remaining 15 subjects. In a standard IFA test, the serum of each of these 24 subjects contained reactive immunoglobulin G (IgG) antibody, and 20 serum samples contained IgM antibody. Four of these subjects also experienced concurrent Lyme disease. Blood samples also were obtained from 91 adult residents of New England who lacked clinical and serological evidence of babesial infection. All blood samples were obtained from study subjects between 1991 and 1997. In all cases, serum was separated from whole blood within an hour of collection, aliquoted into polyethylene tubes, and frozen at −80°F until testing.

Immunoblot assay for anti-*B. microti* antibody. *B. microti* immunoblot kits were provided by Immunetics, Inc., Cambridge, Mass. *B. microti* antigen used in the kits was derived from *B. microti* (GI strain) isolates that were obtained from experimentally infected hamsters. Hamster erythrocytes were separated from whole blood by differential centrifugation, washed and resuspended in Alsever's solution followed by Hanks buffered salt solution, and finally resuspended in distilled water. Erythrocytes were solubilized by addition of polyacrylamide gel electrophoresis (PAGE) sample buffer containing sodium dodecyl sulfate and heating at 88°C for 40 min (10). Solubilized proteins were resolved by electrophoresis on a 10.8% acrylamide–sodium dodecyl sulfate gel followed by electroblotting onto nitrocellulose membranes (10, 18). The nitrocellulose mem-

TABLE 1. Immunoblot reactivity of the sera of subjects who had experienced various diseases or infection

Infection diagnosed	No. of sera	% of sera with reactive band (kDa) present[a]								
		34	36/37[b]	39	53	54	57	60	69	73
Babesiosis	34	62	65	88	82	74	79	79	65	56
Nonbabesiosis (total)	91	1	0	2	2	0	4	0	1	0
Lyme disease	28	0	0	4	8	0	4	0	4	0
HGE	12	0	0	0	0	0	0	0	0	0
None	51	1	0	1	0	0	2	0	0	0

[a] Sera from subjects with babesiosis also reacted against immunoblot bands 122, 102, 85, 72, 71, 70, 59, 58, 46, 45, and 42.
[b] Band at 36 to 37 kDa.

branes were rinsed in phosphate-buffered saline (PBS)–Tween followed by distilled water, dried, and cut into identical 3-mm-wide strips.

The *B. microti* immunoblot kits were comprised of nitrocellulose membrane strips, goat anti-IgG-alkaline phosphatase conjugate, bromo-chloro-indolyl-phosphate–nitroblue tetrazolium substrate, and wash and sample dilution buffer. Serum samples were tested following the kit package insert instructions. The assay procedure consisted of a 30-min incubation of strips with 100-fold dilutions of sera, followed by three buffer washes, a 15-min incubation with conjugate, two buffer washes and two washes in distilled water, a 5- to 10-min incubation with substrate, and a final wash with water. Immunoreactive bands were identified by comparison with bands on a positive-control strip.

Immunofluorescence for anti-*B. microti* antibody assay. Babesial infection was diagnosed serologically by an IFA as previously described (2, 8, 9). Test sera were diluted 1:32 in PBS. The secondary antibody was fluorescein isothiocyanate-labeled goat anti-human immunoglobulin (Kirkegaard & Perry, Gaithersburg, Md.) diluted in PBS with 0.001% Evans blue. Slides were examined at a 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 that reacted at a dilution of 1:32 or greater.

PCR assay for *B. microti* DNA. Whole-blood samples were analyzed and processed by personnel blinded to the clinical status of the donor, as described (6, 13). Barrier-filtered pipette tips and a dedicated set of pipettors were used to prepare all samples. Isopsoralen sterilization was used routinely, and rigorous precautions were used to protect amplification products from contamination. A 238-bp portion of the *B. microti* 16S-like gene was targeted for amplification using a PCR protocol described previously, except that the volume of blood analyzed was 0.5 ml rather than 0.2 ml (6, 13). Control samples included with each amplification assay consisted of three blank control samples with 5 μl of water substituted for DNA and a positive control sample with 60 pg of total *B. microti* DNA (GI strain). Because amplification products were rarely seen on ethidium bromide-stained gels, samples were therefore considered positive on the basis of signal detection after hybridization with a radiolabeled or chemiluminescent internal oligonucleotide probe.

RESULTS

First, we evaluated the sensitivity of the immunoblot procedure for diagnosing human infection due to *B. microti*. Each of the 14 individual serum samples and the 10 paired serum samples from the 24 subjects who were infected by this parasite reacted against 1 or more of 20 *B. microti* proteins on the immunoblot strips, and all reacted against at least one of nine strongly immunoreactive *B. microti* proteins (Table 1; Fig. 1). Only three of the sera failed to react against at least two of these nine bands; two were obtained less than 2 weeks after the onset of symptoms, and one was obtained at 18 months after the onset of symptoms. Reactivities against these proteins were similar in subjects infected solely by the agent of babesiosis and those who were coinfected by the agents of babesiosis and Lyme disease. Reactivity against the immunoblot bands is independent of the severity of babesial illness because the sera obtained from four subjects who experienced asymptomatic

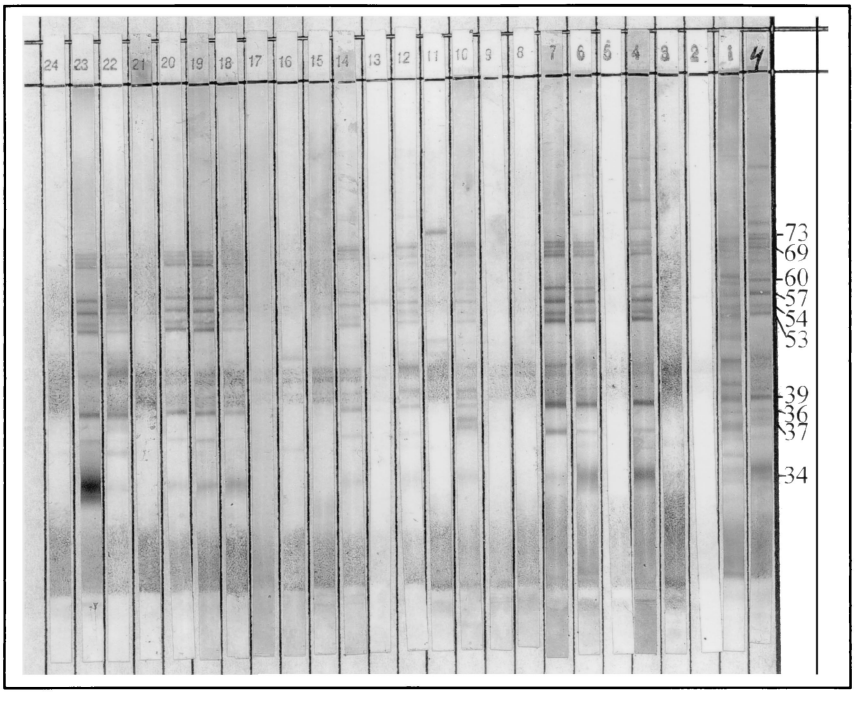

FIG. 1. *B. microti* immunoblot results in subjects with babesiosis and control subjects. Immunoblots were incubated with sera from study subjects as follows: those with babesiosis within the previous 2 weeks (blots 3, 4, 6, 10, and 16), those with babesiosis within the previous 1 to 3 months (blots 1, 7, 12, 14, 20, 22, and 23), those with babesiosis (time of onset uncertain) (blots 18 and 19), those with Lyme disease (blots 11 and 17), and those with no tick-borne illness (blots 2, 5, 8, 9, 13, 15, 21, 24). A molecular weight control (in thousands) is shown in blot 4, at the far right.

TABLE 2. Performance criteria for an immunoblot assay[a]

Diagnostic criterion	% of sera showing no. of reactive bands		
	1	2	3
Sensitivity	100	96	92
Specificity	96	99	100
Predictive positive	86	96	100
Predictive negative	100	99	98

[a] This assay included the sera of 24 residents of New England who experienced episodes of human babesiosis and whose sera were reactive against *B. microti* antigen using an IFA and sera of 91 residents who did not experience babesiosis and whose sera were not reactive against *B. microti* antigen using an IFA.

babesial infection reacted vigorously against two or more immunoblot bands. The presence of serum antibody against the agent of Lyme disease does not alter immunoblot reactivity of sera containing *B. microti* antibody.

To evaluate specificity, we applied the immunoblot procedure against 91 serum samples taken from residents of New England who lacked clinical and IFA serological evidence of babesial infection. These included samples from 51 subjects who also lacked clinical or serological evidence of Lyme disease or HGE, 28 samples from adults who experienced symptoms characteristic of Lyme disease and whose convalescent seroreactivity increased fourfold against *Borrelia burgdorferi* antigen, and 12 samples from subjects who had experienced clinical episodes of HGE and whose seroreactivity increased fourfold against *Ehrlichia equi* antigen. The serum from only 1 of these 91 subjects reacted against two or more of the nine strongly immunoreactive *B. microti* proteins. Sera from these control subjects reacted to only five of the nine proteins. Specificity, using two or more bands as the criterion for a positive test result, therefore, was 99%. Using two or more bands as the criterion for a positive test result, therefore, we conclude that the sensitivity and specificity of the immunoblot assay for diagnosing human babesiosis are 96 and 99%, respectively, with seroreactivity becoming evident within 2 weeks of the onset of symptoms. Similar results were achieved when we compared immunoblot results with those of the IFA (Table 2). The *B. microti* immunoblot assay is sensitive and specific.

Finally, we determined whether the number of reactive immunoblot bands in sera obtained from subjects changed during the course of babesial infection. A positive test (two or more reactive immunoblot bands) was noted in seven of the nine serum samples obtained within 2 weeks of the onset of symptoms and in all 18 of the samples obtained 1 to 3 months after symptom onset. Specimens obtained 6 and 18 months after the onset of symptoms were positive and negative, respectively. Of the serum samples from the five subjects in whom the time of onset of illness was uncertain, all contained IgM IFA antibody and all reacted against two or more immunoblot bands. Specific anti-*B. microti* antibody is produced within 2 weeks after the onset of babesial symptoms and may not be detectable after 18 months.

DISCUSSION

Diagnosis of human babesiosis frequently relies on serological testing. An IFA, using antigen in the form of infected erythrocytes derived from hamsters, provides a sensitive and specific measure of seropositivity and currently remains the method of choice (2, 8). Such IgG and IgM tests are available commercially and are suitable for routine clinical diagnosis of acute *B. microti* infection (8, 9). Although antibody against diverse *Babesia* and *Plasmodium* species may react in this IFA, such reactions generally require relatively concentrated serum samples, at a dilution of no more than 1:16. The usefulness of this IFA, however, is limited because its application is so time-consuming and because its use requires the services of a trained microscopist.

Diagnosis by means of immunoblot testing enjoys important advantages over IFA testing. The immunoblot test can be conducted by generalist technicians, and it may more readily be standardized than can an IFA. The sensitivity and specificity of the *B. microti* immunoblot test that we evaluated are comparable to those of conventional IFAs (8). The immunoblot assay for IgG antibody appears to be well-suited for routine clinical use, because it detects babesial antibody in at least three-quarters of subjects sampled within 2 weeks of the onset of the symptoms of this disease and in virtually all subjects tested thereafter. Furthermore, the immunoblot assay permits discrimination between particular antibodies elicited by diverse *B. microti* antigens and permits their temporal sequence of appearance to be defined closely (1, 19).

The development of a highly sensitive recombinant *B. microti* antigen enzyme-linked immunosorbent assay might be coupled with this *B. microti* immunoblot assay in a two-step test procedure similar to that currently used for detecting antibody against *Borrelia burgdorferi* antigens. In an attempt to standardize the approach to serologic testing for Lyme disease, participants of the Second National Conference on the Serologic Diagnosis of Lyme Disease recommended in October 1994 that laboratories use a two-test approach for the serologic diagnosis of Lyme disease. Specimens are first tested by using a sensitive enzyme immunoassay or IFA. Positive or equivocal specimens are then tested with the more specific IgM and IgG Western blot (19). Although such a two-step approach would improve diagnostic accuracy for babesiosis, the addition of a more highly automated enzyme-linked immunosorbent assay screening test might more economically improve test sensitivity. We conclude that our *B. microti* immunoblot assay provides a sensitive and specific diagnostic test for detecting antibody against this pathogen and provides important advantages over the traditional IFA for detecting *B. microti* antibody.

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