

INFECTION WITH A BABESIA-LIKE ORGANISM IN NORTHERN CALIFORNIA

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Abstract Background. Human babesiosis is a tick-transmitted zoonosis associated with two protozoa of the family Piroplasmorida: *Babesia microti* (in the United States) and *B. divergens* (in Europe). Recently, infection with an unusual babesia-like piroplasm (designated WA1) was described in a patient from Washington State. We studied four patients in California who were identified as being infected with a similar protozoal parasite. All four patients had undergone splenectomy, two because of trauma and two for other medical reasons. Two of the patients had complicated courses, and one died.

Methods. Piroplasm-specific nuclear small-subunit ribosomal DNA was recovered from the blood of the four patients by amplification with the polymerase chain reaction. The genetic sequences were compared with those of other known piroplasm species. Indirect immunofluorescent-antibody testing of serum from the four patients

and from other potentially exposed persons was performed with WA1 and babesia antigens.

Results. Genetic sequence analysis showed that the organisms from all four patients were nearly identical. Phylogenetic analysis showed that this strain is more closely related to a known canine pathogen (*B. gibsoni*) and to theileria species than to some members of the genus babesia. Serum from three of the patients was reactive to WA1 but not to *B. microti* antigen. Serologic testing showed WA1-antibody seroprevalence rates of 16 percent (8 of 51 persons at risk) and 3.5 percent (4 of 115) in two geographically distinct areas of northern California.

Conclusions. A newly identified babesia-like organism causes infections in humans in the western United States. The clinical spectrum associated with infection with this protozoan ranges from asymptomatic infection or influenza-like illness to fulminant, fatal disease. (N Engl J Med 1995;332:298-303.)

THE genus babesia comprises approximately 100 species of tick-transmitted protozoa (family Piroplasmorida) that infect a wide variety of wild and domestic animals.^{1,2} Babesial parasites, together with members of the genus theileria, are referred to as piroplasms because of their pear-shaped intraerythrocytic stages.² Only two species, *Babesia microti* (in the United States) and *B. divergens* (in Europe), have been definitively identified as human pathogens. In 1991, a zoonotic babesia-like piroplasm (designated WA1) that is genetically and antigenically distinct from *B. microti* and *B. divergens* was identified in Washington State.^{3,4} Phylogenetic analysis revealed that WA1 is closely related to the canine pathogen *B. gibsoni* and is secondarily related to theileria species.⁴ Theileria species cause severe febrile illness and pharmacologically reversible lymphoproliferation in African and Eurasian cattle.⁴⁻⁷

Little is known about the prevalence of zoonotic infections caused by WA1 or related piroplasms. Before 1991, only two cases of babesiosis acquired in the western United States had been reported, both of which occurred in California in patients who had undergone

splenectomy; the identity of the piroplasm species was not determined in either case.^{8,9} From 1991 to 1993, four additional patients with piroplasmosis were identified in California, all of whom had an influenza-like illness.¹⁰⁻¹³ In this report, we describe the initial genetic characterization of the protozoal parasites observed in these patients. We also report the findings of preliminary seroprevalence studies conducted in two geographically distinct areas of northern California.

CASE REPORTS

Four patients, all men, who became ill in California were studied (Patients 1, 2, 3, and 4). In addition, blood samples obtained from one patient from Minnesota with a confirmed infection with *B. microti* (Patient 5) were studied (Table 1).

All four California patients had undergone splenectomy, two because of trauma, one (Patient 1) for idiopathic thrombocytopenic purpura, and one (Patient 4) because of Hodgkin's disease. None had recently received a blood transfusion. The patients presented initially with influenza-like symptoms characteristic of early infection with *B. microti* (Table 1),¹ and all were given pharmacologic and supportive therapy appropriate for acute babesiosis. The clinical details of their cases were reported recently.¹⁰⁻¹³ Patient 1 was a 24-year-old soldier who had participated in extensive field-training exercises at Fort Ord (Monterey County) and Fort Hunter Liggett (approximately 64.6 km [40 miles] to the southeast), before he became symptomatic. Patient 2 was a 31-year-old Air Force flight engineer who participated in field-training exercises in San Bernardino County 11 days before he became ill. He had also taken two camping trips in the Sierra Nevada mountains (Fresno County) in the month before the onset of disease. Patient 3 was a 36-year-old man who had been temporarily living and working near Lytton Springs (Sonoma County). His symptoms began about 19 days after a tick bite and increased over the ensuing 10-day period before hospitalization. Despite treatment, he had a cardiopulmonary arrest and died one day after hospitalization. Patient 4 was a 41-year-old man from Kern County who was evaluated for a several-day history of influenza-like symptoms. Relevant exposure history included a four-day camping and hunting trip in the Sierra Nevada mountains (Mono County) that ended eight days before he became ill. The patient recovered after a complicated course that included disseminated intravascular coagulation, pulmonary edema, and renal insufficiency. Figure 1 shows a blood smear prepared the day Patient 4 was hospitalized.

Patient 5 was a 62-year-old truck driver from Minnesota who had

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Table 1. Clinical Features of Four Men with Piroplasmosis.

PATIENT NO.	AGE	DATE OF INITIAL EVALUATION	PRESENTING SYMPTOMS	HISTORY OF RECENT TICK BITE	INITIAL DEGREE OF PARASITEMIA	INITIAL HEMATOCRIT	INITIAL WHITE-CELL COUNT	INITIAL PLATELET COUNT*	OUTCOME
					percent		cells/mm ³		
1	24	9/91	Fever, chills, headache, myalgia, weakness, congestion, vomiting, anorexia	No†	1	40	5,600	46,000	Recovered after treatment with clindamycin and quinine
2	31	8/92	Fever, chills, headache, myalgia, arthralgia, fatigue, malaise, vomiting, dark urine	No	28	47	3,500	147,000	Recovered after treatment with clindamycin and quinine
3	36	7/93	Hypothermia, altered mental status, weakness, myalgia, vomiting, diarrhea, abdominal pain, jaundice, dark urine	Yes	54	17	35,000	NA	Died after treatment with clindamycin, quinidine, doxycycline, pentamidine, red-cell transfusion, and hemodialysis
4	41	9/93	Fever, chills, sweats, headache, body aches, nausea, fatigue, dark urine	No‡	12	44	5,300	86,000	Recovered after complicated course involving disseminated intravascular coagulation, pulmonary edema, and renal insufficiency and treatment with clindamycin, quinine, and red-cell exchange transfusion

*NA denotes not available.

†The patient had been bitten by a tick five months before the onset of his symptoms.

‡The patient had a history of a probable bite from an undetermined type of arthropod.

a six-week history of weight loss, fever, and chills. Infection with *B. microti* was diagnosed on the basis of serologic and polymerase-chain-reaction (PCR) analyses (Pruthi RK, Wiltsie JC, Persing DH: unpublished data).

METHODS

Specimen Collection

Serum specimens were available from all patients for indirect immunofluorescent-antibody testing (Table 2). Analyses with the PCR were performed on whole-blood specimens collected in EDTA or citrate anticoagulant. Whole-blood specimens were obtained from Patients 2, 3, and 4 before therapy (Patients 3 and 4) or during therapy (Patient 2) and were cryopreserved in 10 percent dimethylsulfoxide within three days of collection. The whole-blood specimen from Patient 1 was collected at the end of therapy and then stored at 4°C for approximately five months before analysis.

Seroprevalence Studies

Serum specimens were obtained from potentially exposed persons in California for immunofluorescent-antibody testing with WA1, *B. microti*, and *B. gibsoni* antigens. Specimens were obtained in March 1992 from 51 healthy men (age range, 18 to 31 years) stationed at Fort Ord, most of whom were involved in outdoor field-training exercises at Fort Ord and Fort Hunter Liggett. Follow-up serum samples were requested 10 months later from men with elevated WA1-antibody titers ($\geq 1:160$). Specimens were also obtained from 115 current or former residents of a private ranch near Ukiah (Mendocino County), an area in northern California in which Lyme disease is endemic, who had enrolled in 1988 in a prospective study of the incidence of Lyme disease.¹⁴ The specimens were stored at -20°C before serologic testing was performed.

Serologic Testing

Indirect immunofluorescent-antibody testing was performed as described previously.^{4,15} Positive and negative controls were included with each run. All clinical specimens from the patients were read blindly in separate tests by two investigators from one laboratory. In addition, all reactive specimens (titer $\geq 1:160$) were tested for rheumatoid factor, antinuclear antibody, and antibody to *Toxoplasma gondii* and *Borrelia burgdorferi*.

For the seroprevalence study of the Ukiah group, a different laboratory tested all 115 specimens for antibody to WA1 by using indirect immunofluorescent-antibody slides sent from the first laboratory. The specimens were tested for antibody to *B. microti* with commercially prepared *B. microti* antigen. *B. microti* substrate slides (MRL Diagnostics, Cypress, Calif.) were prepared with *B. microti*-

infected hamster erythrocytes, fixed in acetone, and stored at -20°C before use in the immunofluorescent-antibody procedure. A group of 20 specimens (including the 4 seroreactive specimens) was tested blindly in the first laboratory according to the procedure outlined above, with 100 percent concordance in results between the laboratories.

Genetic Analysis of Piroplasm-Specific Ribosomal DNA

The piroplasm-specific nuclear small-subunit ribosomal DNA (nss rDNA) was recovered from whole-blood specimens by broad-range PCR amplification and analyzed by direct sequencing of the amplification product according to a procedure described previously for *B. microti* and the WA1 piroplasm.^{3,16,17} DNA sequence analysis was performed with broad-range and piroplasm-specific sequencing primers in a DNA-cycle sequencing protocol (GIBCO-BRL, Gaithersburg, Md.) performed on both strands. Sequence construction, alignment, and phylogenetic analyses were performed as described previously.⁴ Two methods were used for phylogenetic analysis: neighbor-joining bootstrap analysis was done with the IBM PC program NJBOOT, and parsimony analysis was done with the PAUP3.0q program on a Macintosh computer as described previously.⁴

RESULTS

Molecular Characterization of the Piroplasm

Attempts to recover the piroplasm by hamster inoculation and by in vitro cultivation of blood specimens from Patients 2, 3, and 4 were unsuccessful (data not shown). We then sought to recover piroplasm-specific nss rDNA sequences by broad-range PCR. A potential complication of this approach to the detection of eukaryotic pathogens is the coamplification of host rDNA sequences.¹⁶ After amplification a piroplasm-specific nss rDNA product (591 base pairs[bp]) was observed for all four California patients (Patients 1, 2, 3, and 4) and a patient infected with WA1 (Fig. 2A).^{3,4} In Patients 1 and 2 (Fig. 2A, lanes 5 and 6) and in the uninfected control, a 651-bp human nss rDNA product was also observed. However, differences in size and sequence composition between the piroplasm PCR product and the human genomic product allowed us to recover and sequence a 1272-bp portion of the

piroplasm-specific gene from the blood of Patient 1 and a 591-bp fragment from the blood of Patients 2, 3, and 4.

Sequence analysis (not shown) revealed that the organisms in the blood of all four California patients were nearly identical (99.8 percent homology). The organisms from two patients (Patients 1 and 3) had identical sequences within a 591-bp region that is highly polymorphic among piroplasm species analyzed to date; this sequence differed by only one nucleotide from the homologous segment recovered from the other two California patients. Phylogenetic analysis was performed on nss rDNA segments recovered from the blood of the four patients; included in the analysis were related sequences from the three known human pathogens (*B. microti*, *B. divergens*, and WA1) and six animal pathogens (*Theileria parva*, *T. annulata*, *B. gibsoni*, *B. rodhaini*, *B. canis*, and *B. equi*) for which sequences were recently determined by us or identified in the Genbank sequence data base.^{4,18} A distantly related apicomplexan, *Tox. gondii*, was used to root the genetic-distance tree (Fig. 2B). On the basis of this analysis, the California piroplasms are most closely related to

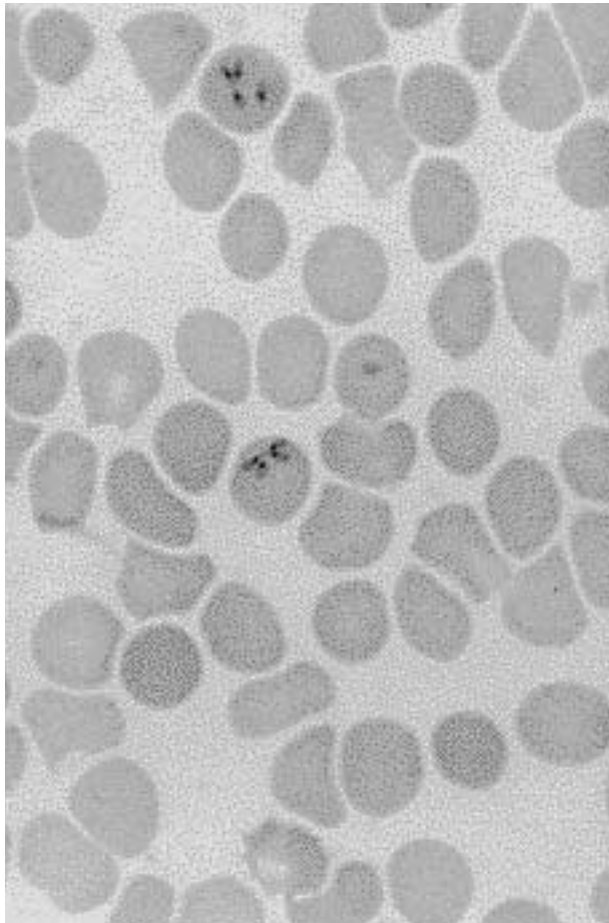


Figure 1. Photomicrograph of a Peripheral-Blood Smear from Patient 4.

Intraerythrocytic tetrad ("Maltese cross") forms composed of four pear-shaped intraerythrocytic merozoites are shown and were present in most microscopical fields. Also present were single-ring forms of intraerythrocytic merozoites. ($\times 700$.)

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Table 2. Indirect Immunofluorescent-Antibody (IFA) Testing of Serum from Patients with Piroplasmosis, 1991 through 1993.

PATIENT NO.	PROBABLE SITE OF EXPOSURE	DATE OF SPECIMEN COLLECTION	IFA TITER TO <i>B. MICROTI</i>	IFA TITER TO WA1 PIROPLASM
1	Monterey County, Calif.	9/91	$\leq 1:80$	1:5120
		3/92	$\leq 1:80$	1:1280
		9/92	$\leq 1:80$	1:640
		4/93	$\leq 1:80$	1:640
2	San Bernardino County or Fresno County, Calif.	9/92	$\leq 1:80$	1:5120
		11/92	$\leq 1:80$	1:1280
		2/93	$\leq 1:80$	1:320
3	Sonoma County, Calif.	7/93	$\leq 1:80$	$\leq 1:80$
4	Mono County or Kern County, Calif.	9/93	$\leq 1:80$	1:5120
5	Crow Wing County, Minn.*	9/92	1:2560	1:160

*The patient lived in a rural area of northern Minnesota but traveled extensively in the United States.

WA1 and to the canine pathogen *B. gibsoni*. The latter group falls into a phylogenetic cluster that includes *B. equi* and theileria species, whereas the other known human pathogens, *B. microti* (from the United States) and *B. divergens* (from Europe), segregate into successively more remote clusters (Fig. 2B). This confirms our earlier observations and those of others of substantial genetic diversity among the zoonotic piroplasms.^{4,18} Moreover, the data provide evidence of a phylogenetic link between the zoonotic piroplasms found in the western United States and lymphotropic piroplasms of the genus theileria, even to the exclusion of some members of the genus babesia itself (especially *B. divergens* and *B. canis*).⁴

Serodiagnosis of Piroplasm Infection

Consistent with the phylogenetic relatedness of WA1 to the piroplasms isolated from Patients 1, 2, 3, and 4, we found that WA1 antigen could be used in an immunofluorescent-antibody assay to assess the serologic responses of these patients. Titers of antibody to WA1 antigen were markedly elevated (1:5120) in three of the four patients within one month after they became ill and then declined appreciably in the following months to 1:640 and 1:320 in the two patients who were monitored (Patients 1 and 2, respectively) (Table 2). Because Patient 3 died soon after hospitalization, only serum samples from the acute phase of the illness could be tested, and they were nonreactive to WA1. Serum samples from the three patients seroreactive to WA1 were also reactive to *B. gibsoni*, albeit at fourfold lower titers (data not shown). Consistent with the phylogenetic and antigenic dissimilarity of WA1 and *B. microti*,^{3,4} none of the serum samples from the California patients showed cross-reactivity to *B. microti*. Serum samples from Patient 5, the Minnesota man who was infected with *B. microti* (confirmed by PCR and DNA sequencing; data not shown), had a titer of 1:2560 against *B. microti* but limited cross-reactivity (1:160) to WA1 antigen (Table 2).

Seroprevalence Studies

In a preliminary assessment of the prevalence of infection with WA1 or related organisms, we tested serum specimens from 51 soldiers at Fort Ord. Eight of

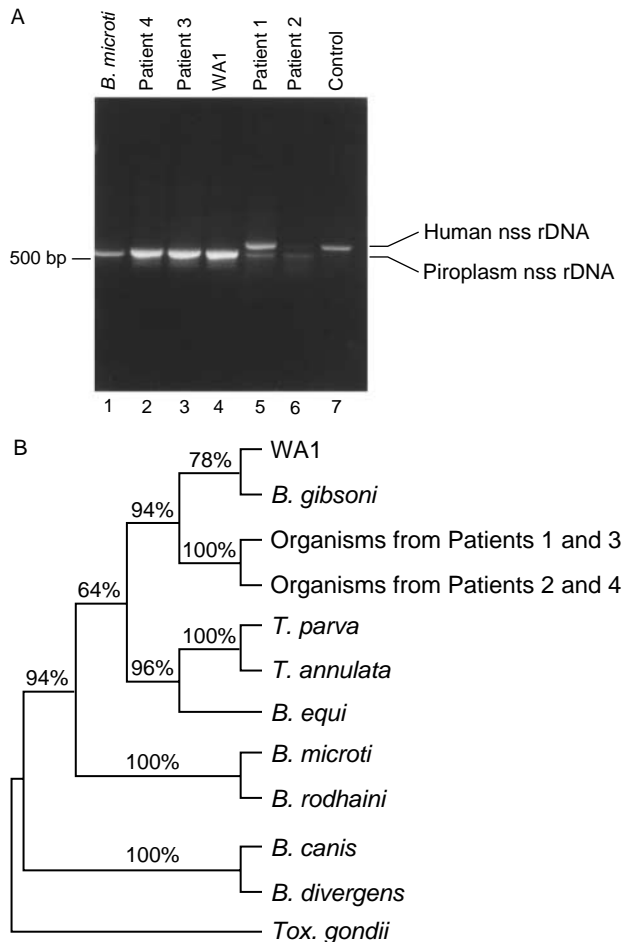


Figure 2. Amplification of nss rDNA Fragments from Whole Blood Collected from Five Patients with Piroplasmosis (Panel A) and Neighbor-Joining Analysis (Panel B).

In Panel A the amplification product from the human nss rRNA gene migrates at 651 bp, whereas the piroplasm-specific product migrates at 591 bp. Lane 7 shows nss rDNA fragments from whole blood from a healthy control without parasitemia. WA1 denotes a patient infected with WA1 piroplasm. In Panel B, neighbor-joining analysis⁴ was performed on 408 alignable nucleotides with 63 phylogenetically informative positions. The percentage of neighbor-joining bootstrap replications greater than 50 percent is shown above each node (branching point); the higher the percentage, the greater the likelihood that the species joined at the right are related. Branch length is not drawn to scale. Analysis of the larger fragment (1272 bp) recovered from Patient 1 gave similar results for a subgroup of the organisms for which additional sequence data were available (data not shown). Although the specific branching order shown is only moderately supported by neighbor-joining bootstrap analysis at some nodes, the level of confidence in the branching order at other nodes is very high. In particular, the cosegregation of *B. divergens* and the domestic canine pathogen *B. canis* from other piroplasm species included in the analysis was supported in 94 percent of bootstrap replications. The piroplasm-specific DNA sequences recovered from Patients 1 and 3 are grouped together because they were completely homologous; the same was true for piroplasm-specific DNA sequences recovered from Patients 2 and 4.

51 enlisted men (16 percent) had elevated titers (defined as a titer $\geq 1:160$) (Table 3). Two of the eight men had WA1 titers of 1:320, the same titer measured in Patient 2 six months after the onset of illness. None of the eight had detectable antibody to *B. microti*, and

none had traveled to an area in which *B. microti* was endemic. However, all of them had had extensive military-related travel within and outside the United States before being assigned to Fort Ord. Four of the eight subjects were retested 10 months after the initial specimen was collected, and three had persistently elevated titers (Table 3). None of these three recalled a recent tick bite.

Another set of serum specimens was obtained from 115 current or former residents of a private ranch near Ukiah who participated in a 1988 study of the prevalence of Lyme disease¹⁴ (Table 3). Four (3.5 percent) had titers of antibody to WA1 antigen of at least 1:160. Follow-up serum samples were obtained in 1992 from two subjects who had elevated titers in 1988. The 1988 and 1992 specimens were tested in parallel, and both subjects had persistently elevated titers of antibody to WA1 antigen. Again, in all four subjects the seroreactivity was apparently specific for WA1; antibody to *B. microti* was not detectable in specimens from these four subjects or from any of the remaining subjects (Table 3). All of the WA1-seroreactive specimens were tested for rheumatoid factor, antinuclear antibody, and antibody to *Tox. gondii*, which might be associated with serologic false positivity or cross-reactivity.¹⁹⁻²¹ The results of these additional studies were largely uninformative (Table 3). Serum from one WA1-seroreactive Ukiah resident was positive for antibody to *Bor. burgdorferi*, which was confirmed by Western blot analysis (Table 3). As a further demonstration of immunofluorescent-antibody specificity, a panel of 36 serum samples from patients known to be infected with *B. microti* showed no cross-reactivity to WA1 (Table 3); some of these patients had titers of at least 1:1024 in response to the homologous antigen. As was observed in the index patients, all of the specimens that were seroreactive to WA1 had limited cross-reactivity to *B. gibsoni* (data not shown).

DISCUSSION

Babesiosis is an emerging vector-borne disease that is endemic in some areas of the northeastern and upper midwestern United States but has been infrequently reported in the western United States. Recently, a novel zoonotic piroplasm (WA1) was isolated from an apparently immunocompetent, normosplenic 41-year-old man from south-central Washington.³ Unlike *B. microti*, the etiologic agent of human babesiosis in the northeastern and Great Lakes regions of the United States, the WA1 piroplasm grew continuously in stationary erythrocyte cultures and had several unique biologic and genetic characteristics.⁴ In this study, analysis of piroplasm-specific DNA recovered from four patients in California showed that the causative agents are related to WA1 and are distinct from the other known zoonotic piroplasms. The agents from the western United States are related to but distinct from the canine pathogen *B. gibsoni*²¹⁻²³ and are secondarily related to theileria species, which cause lymphoproliferative disorders in African and Eurasian cattle.⁵⁻⁷ Taken together, the molecular and immunologic data suggest that WA1 and related organisms

Table 3. Indirect Immunofluorescent-Antibody Testing for Reactivity to *B. microti* (GI/Bm strain), WA1, and Other Antigens in Healthy Persons from Fort Ord and Ukiah, California, and in Persons Infected with *B. microti*.

GROUP ANALYZED	NO. OF SUBJECTS	ANTIGEN TYPE		FOLLOW-UP SEROLOGIC TESTING	ADDITIONAL SEROLOGIC FINDINGS
		<i>B. MICROTI</i>	WA1		
		titer (no. of subjects)			
Subjects from Fort Ord	51	<1:80 (51)	<1:80 (43) 1:160 (6) 1:320 (2)	— At 8 and 10 mo 1 subject had titers of 1:160 and 1 had titers of 1:320 and 1:160, respectively At 8 and 10 mo 1 subject had titers of 1:640	1 subject had a weakly positive antinuclear antibody titer of 1:40 (homogeneous staining pattern); 1 was weakly seroreactive (1:64) to <i>Tox. gondii</i> IgG by indirect immunofluorescent-antibody testing —
Subjects from Ukiah	115	<1:80 (115)	<1:80 (111) 1:160 (3) 1:320 (1)	— At 5 yr 1 subject had a titer equivalent to 1:160 At 5 yr 1 subject had a titer equivalent to 1:320	— 1 subject was seroreactive to <i>Bor. burgdorferi</i> IgG by enzyme-linked immunosorbent assay and Western blotting Not done
Persons infected with <i>B. microti</i>	25	≥1:1024 (25)	<1:80 (36)*	Not done	

*Eleven additional patients infected with *B. microti* were tested for WA1 seroreactivity.

represent a newly recognized species or a group of related species that are distinct from the other piroplasms known to infect humans.

The antigenic cross-reactivity of WA1 with genetically related organisms allowed us to perform seroprevalence studies of zoonotic piroplasmosis among potentially exposed persons, similar to those done for *B. microti*.²⁴⁻²⁶ Various seroprevalence rates were observed: 3.5 percent among persons living in an area of northern California in which Lyme disease is endemic and 16 percent among soldiers stationed at Fort Ord. Three of the subjects tested had titers of 1:320, the same level recorded in Patient 2 six months after his illness. The cutoff titer we used to define a reactive result (1:160) was based on our previous experience with a similar test for *B. microti*^{4,15} and on serial testing of specimens from two patients (Patients 1 and 2) for whom follow-up serum samples were available. However, the serologic results must be interpreted with caution because of uncertainty about the specificity of the methods used.

Although the selective reactivity of serum samples from 12 subjects to WA1 (and its relative *B. gibsoni*) but not to *B. microti* argues against a purely nonspecific mechanism, the ultimate determination of the cutoff titer to be used as an indicator of past exposure must await additional prospective studies. Even if the immunofluorescent-antibody test is highly specific, we cannot determine when or where seroconversion occurred. Although none of the seroreactive subjects had traveled to an area in which *B. microti* was endemic, the subjects from Fort Ord had had extensive military-related travel within and outside the United States. The persistently elevated antibody titers in some of these subjects might be due to chronic subclinical or self-limited infection, reexposure to the pathogen, or other, nonspecific factors.²⁴⁻²⁶

Although an exoerythrocytic stage^{6,7,27} has not yet been found for the organisms described here, such a stage, as shown for *B. equi* and suggested for *B. microti*,²⁷ might serve as a reservoir of persistent infection that is relatively protected from immune surveillance.

A chronic carrier state has been described in animals infected with many species of babesia and theileria, the detection of which may be facilitated by the use of sensitive molecular diagnostic tests.^{16,17,28,29} Recently, persistence of piroplasm-specific DNA has been observed in blood samples from patients in the northeastern United States with previously unrecognized *B. microti* infection.³⁰ The latter findings may constitute the first direct evidence in support of previous seroprevalence studies indicating that chronic subclinical infection also exists in humans.

An arthropod vector for the organism described here has not yet been identified. All piroplasms studied to date are tick-transmitted; *Ixodes pacificus*, which serves as the predominant vector of the Lyme disease spirochete (*Bor. burgdorferi*) in the western United States,^{31,32} can transmit *B. microti* to animals.³³ However, the relatively low seroprevalence rate (3.5 percent) in the Ukiah residents, of whom nearly 25 percent were seropositive for *Bor. burgdorferi*,¹⁴ might be consistent with an independent mode of transmission. One of the four patients described here (Patient 3) recalled being bitten by a tick about 19 days before he became symptomatic; at the time of year that he was bitten, nymphal stages of both *I. pacificus* and *Dermacentor occidentalis* are common in the area (Lytton Springs, Sonoma County) (Clover J: personal communication). A fuller understanding of the risk of human piroplasmosis in the western United States and other areas will depend on the identification of the animal reservoirs of infection and further characterization of the transmission cycle of the etiologic agents.

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