DIAGNOSIS OF TICK-BORNE DISEASES: B. BURGDORFERI, B. MICROTI, AND ANAPLASMA PHAGOCYTOPHILUM

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Draft background paper written to stimulate discussion for the Institute of Medicine Committee on Lyme Disease and Other Tick-Borne Diseases: The State of the Science workshop entitled:
"Critical Needs and Gaps in Understanding Prevention, Amelioration, and Resolution of Lyme and Other Tick-Borne Diseases: The Short-Term and Long-Term Outcomes"
Washington, D. C., October 11-12, 2010

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DIAGNOSIS OF TICK-BORNE DISEASES: *B. BURGDORFERI, B. MICROTI*, AND *ANAPLASMA PHAGOCYTOPHILUM*

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ABSTRACT

Lyme disease is the most common tick-borne zoonotic infection in the northern hemisphere. It is a complex multisystem disorder that may involve single or multiple organ systems. Infection with the causative agent, the spirochete *Borrelia burgdorferi* may involve the skin, central and peripheral nervous systems, as well as targets within the cardiovascular and musculoskeletal systems. Patients at risk for Lyme disease are also at risk acquiring of infection or coinfection with other agents transmitted by the same tick vector, including *Babesia microti* and *Anaplasma phagocytophilum*. Since infection with all three agents can manifest initially as a nonspecific febrile illness with or without specific organ system involvement, the clinical diagnosis of a presenting patient can be challenging. Laboratory methods for detection and discrimination between these infections are of significant value in the clinical evaluation of such patients. This review will describe the curious convergence of these tick-borne infections and the diagnostic challenges they pose, and will detail the laboratory procedures that can be used to decipher the complex array of diagnostic possibilities.

INTRODUCTION

Although significant progress has been made over the past several decades in understanding the immunobiology of Lyme disease and in increasing awareness that this disease is an important public health problem, much about the disease still remains puzzling. Its pathogenesis is still poorly understood, and the interdependent problems of diagnosing the disease accurately and assessing therapeutic outcomes confound one another. One of the most curious aspects of Lyme disease in humans has been its inconsistent presentation, in terms of both disease severity and organ system involvement (1-3). By far, the most consistent finding is erythema migrans, often accompanied by a nonspecific febrile illness. However, even these findings may be absent or unrecognized in many cases, and subclinical or self-limiting infections may occur in a substantial proportion of exposed persons. Within a few weeks to months of infection, a wide array of signs may appear that affect certain subsets of infected patients. These signs (in roughly descending order) include arthritis, lymphadenopathy, meningitis, cranial neuritis (including Bell's palsy), myopericarditis, nonexudative sore throat, mild transient or recurrent hepatitis, and, less commonly, pancarditis, ocular involvement, and adult respiratory
distress syndrome. Years after the onset of disease, occasional patients may develop migratory musculoskeletal disorders along with persistent malaise and fatigue or chronic encephalomyelitis(4). These complications usually respond to therapies directed against *B. burgdorferi*, but some patients have delayed responses and others do not respond at all.

In the United States, the apicomplexan blood parasite *B. microti* and the more recently recognized agent of human granulocytic anaplasmosis (HGA) are also transmitted by the deer tick (5-8). Both infections appear to be most intensely enzootic in established Lyme disease-endemic habitats, and Lyme disease coinfection with one or both of these organisms has been described in most of these areas in the upper Midwest and northeastern United States. In general, cardinal symptoms of babesiosis and anaplasmosis are often absent in coinfected patients, such that coinfected patients can be difficult to distinguish objectively from patients with uncomplicated Lyme disease. However, because the three infections may require different approaches to treatment, an understanding of the natural history of infection with *Babesia* and *Anaplasma* may become critical for understanding the biological variation of human Lyme disease and determining optimal medical management for affected patients. For these and other reasons, considerable effort in several laboratories has gone into further defining the transmission cycles of these agents, developing serological and molecular markers of infection for all three pathogens, propagating all three pathogens from human sources, and defining the extent of human exposure to these pathogens (Reviewed in (9)).

Several other tick-borne pathogens have been described in recent years, including the agent of human monocytic ehrlichiosis (Reviewed in (10) (11, 12)) and a novel babesia species (*B. duncani*) which is found primarily in the western US (13, 14). However, these organisms do not appear to be enzootic with Lyme disease and are not part of the Lyme disease transmission cycle. As a result, coinfection of these organisms with *B. burgdorferi*, though not impossible, is much less likely. The diagnosis of infection with these organisms should be considered on the basis of likelihood within their respective endemic areas, but the focus of this review will be on agents that are proven to be part of the Lyme disease transmission cycle.

**B. MICROTI INFECTION**

*B. microti* infection has been diagnosed in residents of many areas in the northeastern and northern midwestern United States where Lyme disease is prevalent (15-18). It is interesting to note that *B. microti* was once considered to be a possible agent of Lyme disease, since many patients with babesiosis presented with an erythema migrans-like rash at the site of a deer tick bite. The initial symptoms of both illnesses somewhat overlap: like Lyme disease, babesiosis in humans often presents as nonspecific symptoms including fever, fatigue, and other flu-like symptoms. Hemolytic anemia, which lasts from several days to a few months, may occur in patients with clinically severe cases, most commonly asplenic or elderly patients. However, most cases of human babesiosis in normosplenic, immunocompetent patients are probably subclinical and occur as a self-limiting illness (19, 20).

Seroepidemiological data suggest that ~10% of Lyme disease patients in Connecticut and perhaps even higher proportions of such patients in other areas have been exposed to *B. microti* (21, 22). However, these two studies did not provide proof of coinfection (vs. sequential infection) with both agents, because they were conducted retrospectively, and specimens for the direct demonstration of both pathogens were not collected. A prospective study by Krause et al.
(19) provided more convincing evidence of simultaneous infection with \textit{B. burgdorferi} and \textit{B. microtii} in substantial numbers of patients with presumptive Lyme disease from coastal New England. Although not usually recognized as coinfected on clinical grounds, patients harboring both organisms often had more severe disease and a higher likelihood of persistent post-infectious fatigue.

Since antibiotic therapy for early Lyme disease is unlikely to be effective against coinfection with \textit{B. microtii}, it is easy to envision a scenario in which underlying babesiosis is responsible for the persistence of symptoms after therapy for early Lyme disease. However, since the clinical presentation is nonspecific, it will be difficult to know at the time of presentation whether a patient would benefit from therapy targeting babesial coinfection. The risks associated with antibabesial therapy such as atovaquone plus clindamycin are not insignificant, and given a low probability of coinfection, presumptive diagnosis and therapy would not appear to be warranted even in the most heavily endemic areas. It is important to note that the converse scenario does not appear to be true: among patients with case defined chronic fatigue syndrome and no history of antecedent Lyme disease, there is no evidence supporting a major role of coinfection with \textit{B. burgdorferi} and/or \textit{B. microtii} (23). A more conservative approach would employ selective testing of patients, who based on clinical, epidemiological, and initial laboratory evaluations, may be at higher risk of coinfection (see below).

**HUMAN GRANULOCYTIC ANAPLASMOSIS**

In 1994, Bakken et al. (24) described a nonspecific febrile illness among patients in the Upper Midwest that was characterized by thrombocytopenia, and neutrophilic inclusions (morulae). Genetic and serological analyses of patients’ blood samples indicated that the agent of human granulocytic ehrlichiosis was closely related to \textit{Ehrlichia equi} and \textit{Ehrlichia phagocytophila}. Infections caused by a similar or identical agent were subsequently described in many states including New York, Connecticut, Massachusetts, Rhode Island, Minnesota, Wisconsin, and California (Reviewed in (10) (11, 12)). Since then, phylogenetic analyses have determined that the agent of human granulocytic ehrlichiosis is more closely related to the genus \textit{Anaplasma} and the infection is now referred to as human granulocytic anaplasmosis (HGA) (11).

The report of a case of HGA that occurred following a deer tick bite (5), together with recognition of the apparent overlap between areas where Lyme disease and HGA are endemic, prompted an investigation into whether the deer tick could also transmit HGA. \textit{Ixodes} ticks collected from fields in several locations in Wisconsin where HGA cases have been described were analyzed retrospectively; the collection obtained from Wisconsin in 1993 and an earlier collection stored in alcohol since 1982 both contained PCR-positive specimens. Just as retrospective epidemiological studies of \textit{B. burgdorferi} demonstrated the presence of pathogen DNA in suitable vectors (25) or reservoirs (26) from antiquity, the existence of the agent of HGA clearly preceded the first descriptions of the disease itself. Indeed, the increased incidence of HGA is likely to be due, in part, to the emergence of its recognition. In a subsequent study, Telford et al. (8) showed that the deer tick is competent to transmit an agent of HGA that was recovered from a patient on Nantucket Island. \textit{Ixodes pacificus} may be the vector of HGA in the western United States; this tick is the primary vector of \textit{E. equi}, which is closely related to the agent of HGA.

Immunomodulatory effects of \textit{Babesia} and \textit{Anaplasma}: Impact on diagnostic markers?
The elucidation of the immunologic interplay of the microbial agents that coevolve during the transmission cycle of Lyme disease is likely to yield a better understanding of what can be expected in occasional human cases. In the case of multiple pathogens that coexist within a rodent reservoir involved in overlapping transmission cycles (as is the case for B. burgdorferi, B. microti, the HGA agent, and perhaps other organisms), it is reasonable to hypothesize that immune responses to one organism may have an impact on concurrent infections, especially if the underlying infection is associated with immune suppression. Infections by both Babesia and Anaplasma species are associated with substantial immunosuppressive or immunomodulatory effects (Reviewed in (9)). Coinfection by the agent of HGA and B. burgdorferi or by B. microti and B. burgdorferi could have substantial theoretical effects on the immune response, but the practical questions are these: Are disease outcomes altered, and are immunologic effects severe enough to alter immune responses so that they can no longer be used as reliable indicators of infection?

Animal models are helpful in answering this question. Consistent with the above predictions, experimental coinfection of a mouse model with B. burgdorferi and A. phagocytophilum increased the number of CD4+ cells and drove cytokine release toward a Th1 lymphocyte response (27). In another animal model, coinfection with B. burgdorferi and A. phagocytophilum led to increased pathogen burden in blood and tissue, and to more-severe Borrelia-induced arthritis than single infection with B. burgdorferi alone (28). However, during coinfection, levels of IL-12, gamma interferon, and tumor necrosis factor in serum were paradoxically decreased whereas levels of IL-6 were elevated. A similar study of coinfection in C3H/HeN mice evaluated the tissue distribution of tick-transmitted B. burgdorferi and A. phagocytophilum infection by quantitative PCR (29). Coinfected animals showed increased spirochetal burdens in multiple tissues but Anaplasma numbers (found primarily in blood) remained constant. Although antibody responses were diminished somewhat for A. phagocytophilum, levels of antibody that developed against B. burgdorferi were not affected.

Although coinfection with B. burgdorferi and B. microti was found to be associated with increased disease severity and greater likelihood of spirochetemia in human subjects enrolled in a blinded clinical evaluation, (7) experimental evidence of increased severity in animal models has been mixed. Initial studies showed immunologic effects of coinfection in some mouse strains but not others. C3H mice showed no evidence of increased severity, but normally Lyme disease resistant Balb/c mice showed an increase in arthritis severity at day 30, along with reductions in levels of immunosuppressive cytokines IL10 and IL13 (30). Another mouse model showed that dual infection with B. burgdorferi and B. microti appeared to follow independent courses, with no apparent increase in Lyme disease severity (31), although mouse strains used and inoculation times differed between the two studies. In both studies, serological responses to both agents appeared to be largely unaffected by coinfection.

Taken together, the data from animal models and human clinical studies suggest that coinfection with either A. phagocytophilum and/or B. microti may have immunological effects during the course of B. burgdorferi infection, but these effects are not in themselves sufficient to completely abrogate cellular or humoral immune responses to the point of complicating the serodiagnosis of Lyme disease. Likewise, the presence of concomitant infection with B. burgdorferi does not appear to suppress immune responses to either the HGA agent or B. microti to the point that they cannot be detected in diagnostic assays. A. phagocytophilum infection may
be associated with false positive serologic responses to *B. burgdorferi*, (32, 33), but not false negative responses.

**Clinical and initial laboratory evaluation of Tick-Borne Diseases**

For patients exposed to ticks in areas where multiple tick-borne pathogens are endemic, it seems reasonable for clinicians to be aware of clinical signs that may be consistent with each infection alone or in combination, especially when patients with Lyme disease fail to respond promptly to antibiotic therapy. Patients with uncomplicated erythema migrans, without systemic symptoms, may require no further laboratory-based evaluation and can be treated presumptively for Lyme disease in many cases. More severe presentations may merit closer scrutiny. Symptoms including nausea and/or vomiting, fever, chills, sweats, severe malaise, and a delayed clinical response to antibiotic therapy for presumptive Lyme disease were characteristic of *Babesia*-coinfected patients (19). Fever, chills, myalgias, and severe headache are characteristic of granulocytic anaplasmosis (12). Carditis has been described as a complication of Lyme disease, but has also been described in patients with HGA without evidence of *B. burgdorferi* infection (34).

Initial laboratory evaluations to be considered include examination of blood smears for the presence of intraerythrocytic inclusions (merozoites) typical of babesial infection and granulocytic morulae typical of HGA. However, the sensitivity of blood smear evaluations for immunocompetent, normosplenic patients has not been firmly established and may be relatively low for both diseases (see below). The presence of elevated liver enzymes or other hematologic abnormalities may be especially useful in identifying coinfected patients, since both babesiosis and anaplasmosis have been associated with increases in the levels of alanine aminotransferase. Thromocytopenia may also be present in patients with either disease but it is rarely a feature of uncomplicated Lyme disease. Thus, the presence of thrombocytopenia in patients presenting with early Lyme disease should trigger a suspicion of coinfection with one or the other agent.

**Laboratory Diagnosis of Tick-borne Zoonoses**

In general terms, the available laboratory methods for the diagnosis of the tick-borne diseases described here fall into two categories: 1) Direct methods (microscopy, culture, or PCR, depending on the agent), and 2) indirect methods (detection of organism-specific immune responses). With some notable exceptions, direct methods are generally useful for characterization of acute or active infections, whereas detection of antibody responses is most useful for confirmation of clinical suspicion in post-acute and convalescent phases.

Irrespective of the diagnostic method used, an important consideration is the pre-test probability of infection. Since Lyme disease, HGA and human babesiosis are all characterized initially by seasonal occurrence of a non-specific febrile illness, the likelihood of tick-bite or tick exposure, guided by prevalence information for each of the infectious agents, should be a critical gating item for physician test ordering. Unfortunately *I. scapularis* tick exposure is fairly common, and all three agents have been described in most of the areas endemic for Lyme disease, so laboratory testing plays an unusually prominent role in the diagnosis. However, since all of the laboratory methods currently in use have been shown to give rise to false-positive results, the positive predictive values of each of these methods may reach unacceptable levels, particularly when a multiplicity of tests are ordered. According to a recent treatment guideline
from the American College of Physicians and the IDSA, patients with vague subjective complaints (headache, fatigue, and myalgia in the absence of respiratory symptoms) apart from other risk factors are at low risk for Lyme disease. They recommend against routine testing of such patients because the odds of a false positive result are greater than for obtaining a true positive result (35, 36). Likewise, for patients with nonspecific persistent fatigue without a history of Lyme disease, even if living in or near endemic areas, seroprevalence rates for HGA and B. microti are low and these organisms are unlikely to be implicated (23).

Hematologic Evaluation

The complete blood count (CBC) with a manual differential (direct blood smear evaluation by a medical technologist) can be an extremely useful tool in the initial evaluation of patients with nonspecific presentations. Blood counts and peripheral blood smears along with tests of liver function. Leukopenia, lymphopenia, granulocytopenia, and especially thrombocytopenia are found in HGA. Granulocytopenia is less commonly associated with uncomplicated Lyme disease but has been described in some cases. Anemia is and hemolysis are more common in babesiosis, and especially thrombocytopenia is frequently evident in babesiosis and HGA infections.

Babesiosis and HGA can diagnosed directly by observing organisms on Giemsa-stained smears of peripheral blood. For patients with intact spleens, erythrocytes may show B. microti ring forms on thin blood smears; this proportion may be as high as 80% for asplenic patients (15, 18, 37). In one study (38), most patients coinfected with Borrelia and Babesia species were smear negative for babesiosis at the time of presentation and at all time points thereafter.

Blood smear evaluation has been advocated for the diagnosis of HGA when the index of suspicion is high (11, 12, 39), but detection by this route is unlikely unless the examining technologist is alerted to the possibility of HGA. Moreover, visual inspection of blood smears for the length of time required to find a single morula may be impractical. In one study, a small subset of HGA patients with mild infection had intragranulocytic morulae detected on smears (40), in contrast with symptomatic, untreated patients whose smear results were evaluated after several days of fever (24). The blood smear evaluation for HGA should be carried out within a week of disease onset, as sensitivity is highest at this time (39). Blood samples should be collected prior to administering doxycycline therapy since morulae are eliminated from the blood within 24–72 h after the start of therapy (12). However, the since the sensitivity for detecting A. phagocytophilum is only 25–75%, blood smear evaluation is useful if the results are positive but not particularly helpful if the results are negative.

False-positive results are possible; artifacts such as platelets superimposed on red cells or Howell-Jolly bodies can appear like Babesia, (41); and to an untrained eye, a well-separated nuclear segment within a neutrophil may give the appearance of a morula. Acridine orange stain, which intercalates into double stranded DNA, can help enhance detection of B. microti inclusions in red cells and avoid detection of platelet artifacts.
Direct Detection Methods

*B. burgdorferi*

For direct detection of *B. burgdorferi*, a variety of approaches have been used in the clinical or research laboratories, including microscopic evaluation of tissues, detection of *B. burgdorferi*-specific proteins (by EIA or by immunohistochemistry) or nucleic acids (by PCR), and in vitro cultivation. Direct microscopic detection of *B. burgdorferi* has limited usefulness because of low throughput, low organism abundance in tissues (such as skin biopsies) that are confirmed positive by other methods, and the requirement for silver staining and a skilled operator. Antigen detection assays for detection of *B. burgdorferi* have suffered similar limitations; although *B. burgdorferi* antigens can be detected by immunohistochemistry in tissues and by EIA within specimens from confined anatomical sites like CSF and synovial fluid, where clearance of antigens is limited, their presence in other samples such as urine has been more controversial and has not stood up well to additional scrutiny. In contrast, culture and PCR techniques do appear to be diagnostically useful in certain circumstances and we will limit the remainder of this section to these methods.

Culture-based methods for detection of *B. burgdorferi* were used for the initial establishment of the etiologic basis of Lyme disease, and have been used on and off for decades in the evaluation of various clinical samples for research protocols. However, their use for clinical diagnosis has been hampered by long incubation times, poor sensitivity (with the possible exception of skin biopsies from EM), and limited availability of the specialized growth medium for routine use.

The liquid media currently used for recovery of *B. burgdorferi* are modified versions of the original Kelly medium (45) through various modifications made over time (46) (47) (48). The most commonly used medium (Barbour-Stoenner-Kelly II) is commercially available and is used for direct recovery of spirochetes for clinical specimens including plasma, CSF, whole blood and skin biopsies. Since serum supplementation is required for this medium, one source of variability in the efficiency of spirochete recovery has been the presence of anti-spirochetal antibodies in the serum (49). Cultures are visually inspected by darkfield microscopy or after staining with specific antibodies or intercalating dyes. With newer modified media protocols, spirochetes can be detected within 5-7 days, but they may require incubation for up to three months at 30-34°C.

Recent studies of high-volume inoculation of whole blood or plasma have been surprisingly successful at recovering circulating spirochetes. For many years, rates of recovery of *B. burgdorferi* from blood samples collected from untreated patients with EM had been less than 5–10% (50) (51). However, the inoculum volumes in these studies were generally small, and based on previous studies which showed that circulating bacteria in patients with sepsis are often rare, and that the volume of cultured blood is directly related to culture yield, a team of investigators at New York Medical College investigated the use of high volume inocula of blood or blood fractions. They showed the following: 1) recovery of *B. burgdorferi* was superior from serum or plasma compared to the same volume of whole blood (52) and 2) that the culture yields from plasma were significantly greater than that from serum (53). Recovery of *B. burgdorferi* from high-volume cultures (9 or more ml of plasma) inoculated into a modified BSK II medium was above 40%. However, increasing the volume of plasma from 9 ml to 18 ml for adult patients...
with EM met with diminishing returns; a mere 10% increase in culture yield was observed after doubling the inoculum volume (53).

PCR protocols for detection of *B. burgdorferi* have been in use since the few years after the description of PCR itself. PCR was initially promising as a study tool in studies of the retrospective epidemiology of *B. burgdorferi*, including detection of the organism in suitable tick vectors 30 years prior to the formal recognition of Lyme disease in the U.S. (54), and in suitable animal reservoirs nearly a century earlier (55). However, its track record for dramatically improving the general lot of Lyme disease diagnostics has been more spotty, and in general disappointing (Reviewed in (56), (57), (58, 59)). A variety of chromosomal and/or plasmid targets have been used in the various PCR protocols with varying levels of sensitivity (56), (57), (58, 59).

A number of studies have compared to PCR to culture for detection of spirochetes in skin biopsies. Culture has proven to be roughly equivalent to PCR in several studies with a few exceptions(60) (61) (62) Reviewed in (63) Widely disparate results are likely to be attributable to differences in the various studies in the PCR protocols employed, including type of PCR and/or primer and target selection and/or method of tissue preservation, as well as differences in culture techniques, including size of the skin biopsy sample cultured and/or choice of culture medium. In one study of skin biopsy samples of 47 untreated patients with EM lesions, culture sensitivity was 51%, compared 81% by using a Taqman-based real-time PCR (61).

In contrast to the experience with skin samples, the sensitivity of PCR for detection of *B. burgdorferi* in blood samples has been much lower in patients with EM (64) (65). In a prospective study of U.S. patients with EM, *B. burgdorferi* sensu stricto DNA was detected by PCR in only 14 of 76 (18.4%) plasma samples (64). Detection of the spirochete in blood by PCR in blood samples for patients with disseminated disease has also been disappointing (66) (67). This is likely due to the same issue that hampered early studies of culture in blood samples of Lyme disease patients; specifically, the relative paucity of circulating spirochetes in the small volumes of blood that are typically extracted in a PCR procedure. Most PCR protocols test only 100 to 200 microliter samples and these may simply be inadequate to capture enough circulating spirochetal DNA for detection, even by as sensitive a method as PCR. So it is no surprise that given the relative lack of sensitivity of PCR in blood for detection of early, even disseminated disease, it would fare even more poorly in patients with later disease manifestations, despite positive serological findings in many of these patients (68).

Within anatomically confined compartments, PCR detection methods generally fare better. In a study of 60 U.S. patients with neuroborreliosis (16 with early and 44 with late neuroborreliosis), the sensitivity of PCR in CSF was 38% in early and 25% in late neuroborreliosis, and an inverse correlation was found between duration of antimicrobial treatment and PCR results (69). In this study, four different PCR primer or probe sets were used, three targeting OspA genes and one targeting OspB genes, and concordance between the different assays was relatively poor which suggested low target number within the samples. Other studies from Europe have generated similar findings (70) (71). Studies of synovial fluid, have provided the greatest clinical utility of PCR testing for the diagnosis of active Lyme arthritis and for monitoring the course of therapy (72) (3) (73), (67, 74) (56, 59, 63, 75-77). In a landmark study of 88 patients, *B. burgdorferi* DNA was detected in synovial fluid of 75 (85%) patients with Lyme arthritis (76). Not surprisingly, the PCR positivity rate was lower in patients who had received what should have been effective antibiotic therapy compared to untreated or
undertreated patients. Of 73 patients who were untreated or treated with only short courses of oral antibiotics, 70 (96%) had a positive PCR in synovial fluid samples. In contrast, 7 of 19 (37%) patients who received either parenteral antibiotics or oral antibiotics for more than 1 month were still PCR positive (76). The observation of higher sensitivity of PCR detection of plasmid-encoded OspA compared to multiple chromosomal loci (the 16S rRNA gene and flagellin) in synovial fluid specimens (78) ((76) (75) was referred to as “target imbalance.” It has been speculated that *B. burgdorferi* sensu stricto present in the synovium may selectively shed OspA DNA segments into the synovial fluid (75). Using the same or similar reagents, this phenomenon did not appear to occur in CSF specimens (79).

A subsequent meta-analysis has largely confirmed the above assessment; it showed that PCR is a sensitive diagnostic tool for detection of *B. burgdorferi* DNA in skin biopsy and synovial fluid specimens whereas detection of the organism in blood or blood fractions and in CSF specimens is relatively low (56). Given the paucity of supporting data for use of PCR as a diagnostic technique in samples other than skin and synovial fluid, extreme caution must be exercised in interpreting PCR test results from samples such as whole blood, urine, and cerebrospinal fluid. Even the best PCR laboratories have had problems with PCR amplicon contamination which, if sporadic, can give rise to undetected rates of false positive results which have been reported in a number of PCR assays, including those for *B. burgdorferi*. Because of the high credibility assigned to PCR by most clinicians, false positive PCR results can lead to mistreatment and mismanagement, sometimes with fatal results (80, 81). Laboratories performing PCR should Good Laboratory Practices for controlling amplicon contamination, including strict separation of amplified material from areas where clinical samples are being prepared and inclusion of appropriate positive and negative controls in each run.

**Direct Detection of Babesia microti**

*Babesia microti* has not been successfully grown in culture, despite multiple attempts, and animal inoculation of whole patient via the intraperitoneal route has been the mainstay of organism propagation for many years. This method is used primarily for organism isolation from patients with obvious parasitemia as demonstrated on Wright-Giemsa stained blood films, and it is limited for practical reasons to research laboratories. Hamster blood, which supports replication of *B. microti*, begins to show parasitemia at around day 7 after inoculation, and typically reaches peak levels at 2-3 weeks although samples with low numbers of parasites may take longer.

PCR has gained popularity as a diagnostic method, mainly for two reasons: 1) it provides good sensitivity levels and a broad dynamic range, making it suitable for parasitemic patients who are smear negative as well as smear positive, and 2) its turnaround time and general availability as a diagnostic procedure are far superior to comparable methods based on animal inoculation. Initial descriptions of PCR for detection of *B. microti* were based on conserved elements of the 18S ribosomal gene, and showed that all strains of *B. microti* tested were reactive whereas DNA from *Plasmodium* and other related parasites were negative (82). The 3 hour method was much faster and easily 10 to 100 fold more sensitive than hamster inoculation when tested against a limiting dilution of parasites in whole blood (82). The same PCR assay was then used in several studies of babesial infection and coinfection with *B. burgdorferi* to demonstrate that it was more sensitive than blood smear evaluation for the diagnosis of babesial
infection (83) and that it could be used to detect *B. microti* infection in symptomatic and asymptomatic individuals including blood donors (19, 83-85).

The utility of PCR for detection of *B. microti* was demonstrated in several prospective studies of exposed populations living in Block Island, RI and Eastern Connecticut. Krause et al showed that patients with convincing evidence of simultaneous infection with *B. burgdorferi* and *B. microti* often had more severe disease and a higher likelihood of persistent post-infectious fatigue [7]. Coinfected patients also appeared to be more likely to be spirochetal as determined by detection of *B. burgdorferi* DNA in their blood. In another landmark study (85), Krause et al showed 1) that patients with babesial infection often had prolonged and sometimes persistent parasitemia, as demonstrated by PCR, after primary infection, and 2) that many patients with primary infection with *B. microti* had infections that were subclinical and/or asymptomatic, and which, even if suspected, would not have been detected by blood smear evaluation (85). However, for practical purposes it has been found that virtually all patients who are symptomatic and PCR positive for *B. microti* are already also seropositive (15, 84), so the use of serologic methods, even in the initial diagnosis of babesial infection or coinfection with other tick borne organisms, is not unreasonable (see below).

These studies paved the way for a better understanding of the incidence of chronic, subclinical infection and the potential risk to the blood supply of blood donors who may unwittingly donate parasitemic blood to increasingly compromised populations including transplant patients, cancer patients, and other vulnerable recipients. Several studies have been done using serologic testing followed by PCR testing to identify asymptomatic carriers of *B. microti* in the blood donor population (86, 87). In almost all cases, persons identified as asymptomatic carriers are blood smear negative and thus would have escaped detection upon routine physical examination, yet some of them are positive by hamster inoculation and all are considered potential persistent carriers of *B. microti*. In part due to the recent rise in the number of transfusion related deaths associated with *B. microti* infection, this has led to several new initiatives by the FDA and CDC to institute screening measures by both serologic methods and PCR to detect asymptomatic donors in order to exclude infected units of blood from entering the US blood supply (86, 87). Because PCR may be able to detect parasitemia before serocoversion, it may play a pivotal role in such screening efforts.

**Direct Detection of Anaplasma phagocytophila**

Direct cultivation of Anaplasma phagocytophila was first accomplished by Goodman and colleagues (88) in the few years after the first recognition the disease, by culture of whole blood in the promyelocytic leukemic cell line HL-60. Recovery of *A. phagocytophilum* in cell culture can be used to definitively diagnose infection *A. phagocytophilum* by direct inoculation of cell cultures with peripheral blood from a potentially infected patient (12) (89). The bacteria develop within vacuoles to form morulae in the cytoplasm of infected cells, which can be detected using Wright or Giemsa staining. Intracellular organisms can be visualized as soon as 5 days postinoculation or can remain undetectable for more than 2 weeks. Culture of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of *A. phagocytophilum* in HL-60 cells is arguably the most sensitive method for the direct diagnosis of...
PCR is also a sensitive tool for detecting *A. phagocytophilum*. PCR was used early on to demonstrate the presence of the organism in blood samples and in initial studies that led to the identification of the deer tick vector as a vector (5). Several studies and reviews of the diagnostic performance of PCR for the diagnosis of HGA have been published (58, 92-95). Sensitivity is approximately 67–90% for detecting *A. phagocytophilum* DNA (89), but just as for direct smear evaluation, the PCR sensitivity will likely be affected by the phase of infection and antibiotic therapy. After the first week, the bacteremic phase of infection rapidly wanes, thereby limiting the effectiveness of PCR as a diagnostic technique (39). In general, PCR positivity correlates with the concurrent presence of IgM antibodies and eventual IgG seroconversion.

### Immunodiagnostic Methods

For all of the tick-borne infections described here, the mainstay of the laboratory diagnosis is the detection of antibody responses in serum of affected individuals. Initial detection of organism-specific IgM antibodies during acute illness is accompanied by conversion to IgG during the convalescent phase, usually 10 to 14 days later. Serologic testing is more available than direct detection methods based on culture or PCR, and usually less expensive. Given the relative lack of sensitivity of direct detection methods, serologic testing is also usually more sensitive, especially for detection of *B. burgdorferi* infection. Multiple testing formats are available from commercial sources, including ELISA, IFA, and immunoblotting. This section will provide a summary of immunodiagnostic approaches for each of the three agents addressed by this review.

**B. burgdorferi**

The regional antigenic variability and complexity of the antigenic components of *B. burgdorferi* have posed challenges for the serodiagnosis of LB. In Europe, Lyme borreliosis is caused primarily by the three species: *Borrelia burgdorferi* sensu stricto, *B. afzelii* and *B. garinii*. In the US, substantial genetic heterogeneity of *B. burgdorferi* sensu stricto exists, as assessed pulsed-filed gel electrophoresis typing, but the vast majority of human clinical isolates fall into a few closely related PFGE clusters of strains that have similar antigenic composition (96). *B. burgdorferi* strains used in the US for immunodiagnosis generally are drawn from these groups, so it is likely that sufficient serologic crossreactivity exists among these strains to allow for detection of antibody responses irrespective of the geographic location of the infection (96). However, to complicate things, some immunodominant antigens or epitopes are expressed exclusively in vivo but not in cultures of organisms that are used to prepare antigens for diagnostic testing (97) (98). This has led to the use of serological expression cloning to detect antigens that are expressed in vivo, which in some cases have led to the identification of diagnostically useful reagents.

Several initial studies of the immune response to *B. burgdorferi* recognized the flagellin protein as an immunodominant antigen that was recognized by IgG and IgM antibody subclasses within a few days of infection (99) (100). Unfortunately, the flagellin gene is conserved among multiple species of bacteria, so crossreactivity and nonspecific immune responses are quite common, especially on immunoblots where the protein is denatured (101) (102). Another immunodominant antigen recognized early during infection with *B. burgdorferi* sensu lato is the OspC protein which is encoded on a plasmid plasmid (103). Osp C is upregulated by the spirochete while is still in the tick midgut and when it begins to migrate to the tick mouthparts
prior to transmission. Some strains of *B. burgdorferi* cultured in vitro express little or no ospC; indeed, this antigen was unrecognized in early studies that used high-passage strains as the source of antigen. OspC is genetically and antigenically very heterogeneous (104). Recently, conserved epitopes within the OspC protein have been identified comprising the C-terminal 10 amino acids of OspC (pepC10) (105). Inclusion of this peptide in immunoassays may be an important step toward developing improved immunodiagnostic assays for Lyme disease (see below).

The so-called Vmp-like sequence expressed (VlsE) protein, a hypervariable immunodominant surface lipoprotein encoded by a linear plasmid, was described in conjunction with expression cloning experiments (106-108). Although subject to extensive antigenic variation, presumably because of immunological pressure, within the variable regions a constant domain was identified called IR6 (or C6). Peptide mapping of this region showed that it is highly immunogenic (109). Broad conservation of this region across multiple species of *B. burgdorferi* sensu lato have suggested that it would make a good immunodiagnostic reagent, and several studies have now been published that support its use alone or in combination with other immunodominant peptides. Several other antigens have been identified for potential immunodiagnostic use, including decorin binding protein (DBP1) (110) and a fibronectin binding protein (BBK32) (111).

**Testing Formats**

Over the years, many commercial immunoassays to detect *B. burgdorferi* antibodies have been cleared for use in the United States by the Food and drug Administration (FDA). Most of these assays use the B31 type strain of *B. burgdorferi* as the source of antigen, but several newer assays have employed recombinant antigens and/or peptides. In general, performance of these tests on blinded proficiency surveys of laboratories across the US has been good, but a significant degree of performance variation still exists between test manufacturers and test methods (unpublished observation from 2009 CAP surveys). One potential source of variability is the methods used for antigen preparation. For immunoassays using whole cell preparations, a surprising amount of antigenic variability will be encountered depending on growth conditions, medium used, incubation temperatures, growth phase of the organisms, and methods used to extract antigens, concentrate them and link them to specific substrates for test preparation. Lot-to-lot reproducibility and quality control is a major concern of methods based on whole cell lysates. In contrast, methods based on recombinant proteins or peptides are better defined and may lead to more lot consistency, but the presence of one or a few epitopes poses a different kind of limitation: the intrinsic variability of the human immune response. Thus, justification of the use of a variety of assay formats continues for a variety of reasons ranging from test performance to cost per test. In general, however, efforts to encourage better standardization should lead to better defined immunologic substrates, and challenging proficiency testing programs are one of the best ways to identify the strengths and weaknesses of individual tests and approaches, whether they are user-developed or commercial methods.

Indirect fluorescent antibody tests look for immunoadsorption of patient antibodies to cultured spirochetes attached to glass slides. A dilution series of patient serum placed in adjacent wells, incubated on the substrate for a short time, and then detected by fluorescent microscopy after staining with fluorophore-conjugated -human IgG or IgM antibody. Antibody titers of 1:64 or above are generally considered positive, though there may be considerable day-to-day and
technologist to technologist variation in the determination of values because of the subjective nature of the procedure. Automated fluorescence readers have the potential to improve upon this, but variations in the procedural steps such as preparing the dilution series and washing stringency will continue to contribute to variability.

Enzyme immunoassay (EIA) is the most commonly used method for detection of immune responses to *B. burgdorferi*. Typically, antigen preparations comprising whole-cell sonicates of *B. burgdorferi* are bound to plastic EIA plates for detection of total Ig response or individual antibody subclasses. With EIAs using well-prepared whole cell antigen preparations, about half of patients presenting within a week of the development of erythem migrans will register positive results. In untreated patients after one week, and in patients with multiple EM lesions or signs of systemic involvement, sensitivities of EIA approach 90% (112). EIA is nearly uniformly positive in sera of patients with late manifestations of disease (112).

False positive EIA reactions can and do occur in some patients with bacterial infections due to other organisms; this is thought to be due to crossreactivity with common bacterial antigens such as heat shock proteins, flagellin, and other conserved bacterial proteins (101) (102). Because all commercially available assays using whole cell sonicates contain the ospA protein, individuals who in the past have received the OspA vaccination will often generate false positive results (113) (114). EIAs based on strains that are negative for ospA (by virtue of losing the linear plasmid encoding the protein) or recombinant protein or peptide EIAs devoid of ospA epitopes will be non-reactive in these individuals (115). Because of the lack of specificity of EIA procedures, and because the pretest probability of having Lyme disease is often lower than the false positive rate (especially in non-endemic areas), EIA reactive sera should be confirmed by Western immunoblotting according to so-called two-tier testing guidelines.

Western immunoblotting is performed by carrying out electrophoretic separation of spirochetal antigens from whole cell sonicates, followed by transfer to nylon filters and detection of patient antibody reactivity to the denature proteins on the blot by using labeled anti IgG or IgM antibodies. Several commercial kits for WIB are available which employ a variety of *B. burgdorferi* strains. Choice of the strain and the growth conditions used will likely affect expression levels of many immunodominant proteins, so significant variation has been seen in WIB performance between labs, as determined in proficiency testing surveys (116). Rates of detection of IgM antibodies in patients with EM early in the course of disease have ranged from as low as 3% to as high as 84% (103) (112); some commercially available WIB kits failed to detect IgM responses even after several weeks of infection (117, 118). For detection of IgG antibodies, performance has generally been better, especially for convalescent sera (59) (112). Early IgG responses are dominated by antibody to ospC (assuming the strain used makes sufficient levels of ospC) and flagellin. As mentioned, flagellin responses are the least specific of all the IgG responses for reasons cited above. Later in the convalescent phase, additional reactivities are observed against a range of proteins including BmpA (39 kDa) and B31n protein species at 93-, 66-, 45-, 35-, 30-, and 18-kDa.

Interpretive criteria for IgG and IgM WIB have been established for US patients (119). The IgM criteria of Engstrom et al. (103) were adopted based on a study of early Lyme disease patients; they were established for use only within the first 4 weeks of illness. A positive IgM blot was defined by the presence of two of three reactive protein species in strain 297 (OspC, 41 or 39 kDa). The IgG criteria were based on work from Dr. Alan Steere’s group (112); these were intended for use at any time during the course of illness, although they were expected to be more
informative and applicable to patients later in their disease course. IgG reactivity against at least 5 of 10 protein species (93, 66, 58, 45, 41, 39, 30, 28, 21, or 18 kDa) in strain 39/40 was considered the minimum number to be considered positive.

WIB may be more sensitive than EIA for detection of early Lyme disease, but the two methods are similar in sensitivity for convalescent and late-stage infection. In a study of 46 culture-confirmed patients with EM, IgM WIB was considered reactive in 43% of patients, compared with 33% by EIA (117). This is curiously the opposite of 2-tier strategies used for viral serological testing, in which the first test is more sensitive, the second more specific. Operational aspects preclude the use of WIB as a screening test, however, and other factors may influence the performance of the WIB as a screening test. For example, many of the commercial WIB kits use strain B31, which was included in none of the studies that led to the standard interpretive criteria. Although each kit is supposed to have custom criteria that match the standard criteria, this is subject to interpretive error. Subtle differences in molecular weight of antigens, along with antigen degradation can lead to confusing results, even in experience laboratories. In addition, as with EIA methods, false positives can occur from previous vaccination with the ospA-containing vaccine (113) (114).

Despite this apparent role reversal of screening and confirmatory testing, the recommended use of two-tier testing has helped to resolve much diagnostic uncertainty in the diagnosis of Lyme disease in the US, largely because it improves overall specificity. In most studies of the two-tiered strategy, specificity levels have been 97 to 99%. The low false positive rate has been welcomed, especially in areas where the pre-test probability of infection, especially among patients presenting with non-specific symptoms. However, the reduction of sensitivity for detection of early disease was an unfortunate compromise, especially since non-specific febrile illness can be presenting features of HGA, Lyme disease, and human babesiosis, where diagnostic differentiation is most critical. In 280 patients with various manifestations of Lyme disease, 38% of patients with EM during the acute phase were seroreactive; 67% were positive after antimicrobial treatment (120). The sensitivity increased in patients with evidence of more invasive disease; 87% of patients with early neuroborreliosis and to 97% of those with Lyme arthritis were seroreactive. Similar results were obtained in another study for 47 patients with clinically defined EM, for whom the sensitivities of the two-tier test were 40.4% in acute-phase sera and 66% during the convalescent phase after treatment (61).

Newer antibody tests based on recombinant proteins or immunodominant peptides have the potential of maintaining current levels of specificity without sacrificing sensitivity, especially for detection of early disease. Many studies have been done on recombinant proteins, many of which have been mentioned above, with sensitivities ranging from 30% to nearly 100% depending on the antigen used and the stage of disease being tested reviewed recently in (59). Specificity levels have been somewhat lower than expected (as low as 91%), perhaps because of the presence of small amounts of contaminating protein from the strains of bacteria or yeast used for protein expression. In general, however, no single recombinant protein has served as the universal solution for immunologic detection of both early and late disease (59). Considerably more promise has been shown in combining immunodominant epitopes, especially those that derive from antigens that are recognized early in the course of disease.

In a different study of the same set of 839 serum samples (including those from 280 Lyme disease patients) cited above, antibody responses to recombinant VlsE1, the C6 peptide, and the conserved C terminal peptide from ospC (pepC10) were evaluated by kinetic enzyme-
linked immunoassay (120). At 99% specificity, the overall sensitivities for detecting IgG antibody to rVlsE1 or C6 in samples from patients with diverse manifestations of Lyme disease were equivalent to that of 2-tiered testing. When data were considered in parallel, 2 combinations (IgG responses to either rVlsE1 or C6 in parallel with IgM responses to pepC10) maintained high specificity (98%) and were significantly more sensitive than 2-tiered analysis in detecting antibodies to *B. burgdorferi* in patients with acute erythema migrans. In later stages of Lyme disease, the sensitivities of the in-parallel tests and 2-tiered testing were high and statistically equivalent. In established cases of Lyme disease, the C6 ELISA may also be useful for tracking therapeutic responses (121-125). Although similar observations were made in other studies, some studies have not confirmed these findings (126). Operational differences in the ways the assays were performed may account for this. Taken together, these data are supportive of the goal of potentially replacing 2-tiered testing while improving the diagnosis for early Lyme disease patients.

**Serodiagnosis of Babesiosis**

The serological testing methods for *B. microti* are not as far along as those for Lyme disease, mainly because the organism cannot be propagated in vitro. The mainstay of the laboratory diagnosis is the IFA test, in which a standard 1:16 or 1:32 dilution of serum is dispensed onto wells of a microscope slide that has been previously coated with infected red blood cells, usually of hamster origin. After a wash step, addition of fluorescent anti-human IgG or IgM, and an additional wash, the microscope wells are examined under a fluorescent microscope for staining of intraerythrocytic merozoite forms of the organism. If the screening serologic testing is positive, further 1:4 serial dilutions are performed to determine the serum titer. Determination of titers is somewhat subjective; it is usually associated with a significant, abrupt drop in the level of observed fluorescence. In most laboratories, a 1:64 cutoff titer has been established and is considered to be consistent with exposure to *B. microti*.

In animal models of infection, IgM antibodies precede IgG responses by 7 to 10 days, but in humans, by the time patients present (usually with nonspecific “flu-like” symptoms) they are already IgG positive (15, 84, 127, 128). During the acute phase of infection in which patients are PCR positive, antibody titers typically reach 1: 512, 1:1024 or higher and then decline in the weeks and months after therapy. Even with therapy, however, antibody levels typically persist at 1:64 or above for years after the initial infection. Thus, the finding of an antibody titer at 1:64 during acute presentation should not be assumed to be due to active infection; it may simply reflect past exposure, especially if the patient is form an endemic area. PCR testing may be helpful in resolving this issue. On the other hand, titers of greater than 1:512 have been found to be consistently associated with acute infection and correlated with PCR positivity (85, 128, 129).

Enzyme immunoassays based on antigen preparations from infected hamster erythrocytes have been developed, but have been generally hampered by the presence in of anti-rodent antibodies in serum of a few percent of patients. These confounding antibodies can to some extent be pre-adsorbed prior to testing to reduce this problem. Immunoblots made from partially purified antigens have also been developed (130).

More recently, recombinant expression cloning efforts, using *B. microti* expression libraries screened with human serum, led to the discovery of several recombinant proteins that show promise as immunodiagnostic reagents (130, 131). From two serocomplementary *B.
microti-specific antigens, peptide mapping was done to identify immunodominant epitopes that could be combined in a peptide EIA. The prototype peptide EIA was used to detect B. microti-specific antibodies in 15 sera taken before infection and 107 taken after infection from 59 individuals with known tick-borne infections previously confirmed by other methods. The combination peptide detected 98 out of 107 sera taken after infection that were immunoblot positive; this included 12/12 samples that were PCR positive and six sera from smear-negative patients that were confirmed positive by PCR, immunoblot, or IFA.

In another interesting study, proteins of B. microti that are potentially secreted or surface exposed were identified by serologic expression cloning (132). This report described the identification and initial characterization of 27 clones representing seven genes or gene families that were isolated through serological expression cloning by using a technique that was specifically designed to screen for shed antigens. In this screen, sera from B. microti-infected SCID mice, putatively containing secreted or shed antigens from the parasites, were harvested and used to immunize syngeneic immunocompetent mice (BALB/c). After boosting, the sera from the BALB/c mice, containing antibodies against the immunodominant secreted antigens, were used to screen a B. microti genomic expression library. Analyses of the putative peptides encoded by the novel DNA sequences revealed characteristics indicating that these peptides might be secreted. Initial serological data obtained with recombinant proteins and a patient serum panel demonstrated that several of the proteins could be useful in developing diagnostic tests for detection of B. microti antibodies and antigens in serum. Unfortunately, however, to date none of these recombinant peptides or proteins has been converted into an immunodiagnostic test that is widely available.

Unlike for Lyme disease serology, no proficiency testing programs yet exist for serodiagnosis of B. microti infection, mainly because of lack of demand for such a service. However, this leaves open the possibility that among laboratories performing serodiagnostic methods for this organism, significant inconsistencies may exist between labs and within labs from run-to-run.

Serodiagnosis of HGA

Like for B. microti, detection of antibody responses in patient serum by the indirect immunofluorescence (IFAT) assay is the most frequently used test for clinical purposes (133). However, unlike for B. microti infections, the onset of symptoms after tick bite is fairly abrupt, and symptomatic patients are frequently seronegative. IFA assays typically use HL-60 cells infected with A. phagocytophilum as a substrate, and screening and dilution series are typically performed as described above for B. microti. Sensitivity is high 2–4 weeks following disease onset compared with a few days for PCR, blood smear microscopy and cell culture. A diagnosis of A. phagocytophilum is confirmed by a fourfold increase in antibody titer between acute and convalescent sera or a seroconversion to a titer of 128 or higher (133) (12). Like for B. microti, seropositivity for A. phagocytophilum, often lasts for months and sometimes years after initial exposure (90). Thus, like for B. microti and B. burgdorferi, an HGA antibody titer must be considered in light of other clinical evidence of infection and should not be used as the only criteria for diagnosis. False-positive or cross reactive serologic test results can be seen in cases of Rocky Mountain spotted fever, typhus, Q fever, brucellosis, Lyme disease, Epstein–Barr infection and any variety of autoimmune disorders associated with production of auto antibodies (11). Auger-Rosenfeld et al evaluated the antibody responses by IFA in the sera of 24 patients
with culture-confirmed HGA (90). Patients were followed for up to 14 months. Seroconversion was observed in 21 of 23 patients (91.3%) from whom convalescent-phase sera were obtained. Antibodies were first detected at an average of 11.5 days after onset of symptoms. Peak titers (≥2,560 for 71.4% of patients and ≥640 for 95.2% of patients) were obtained an average of 14.7 days after onset of symptoms. Most patients were still considered seropositive after 6 months, and half of the patients were still seropositive after 11 months.

Some progress has been made toward development of recombinant serological reagents for the diagnosis of HGA, which might be more amenable to automated testing and or rapid immunodiagnostic assay formats(134). Lodes et al described a panel of seven recombinant antigens, derived from the HGA agent, which were evaluated by class-specific enzyme-linked immunosorbent assays (ELISAs) for utility in the diagnosis of the infection. Fourteen genomic fragments, obtained by serologic expression screening, contained open reading frames (ORFs) encoding 16 immunodominant antigens. Eleven of these antigens were members of the major surface protein (MSP) multigene family. In addition to two MSP recombinant antigens (rHGE-1 and -3) and a fusion protein of these antigens (rErf-1), five further recombinants were evaluated by ELISA. Two of these antigens (rHGE-14 and -15) were novel, while a third (rHGE-2), with no known function, had already been described. The final two recombinant antigens (rHGE-9 and -17) represented overlapping segments of the ankyrin gene. When serologic data for all recombinants were combined, 96.2% (26 of 27) of convalescent-phase patient serum samples and 85.2% (23 of 27) of acute-phase patient serum samples were detected, indicating the potential of these antigens for use in the development of a rapid serologic assay for the detection of HGA.

CONCLUSIONS

The three identified infections transmissible by the bite of a deer tick in the United States—Lyme disease, HGA and human babesiosis—have distinct and overlapping clinical features that, in the case of infection by more than one organism, may confound each other. Each may require different treatment approaches, although one can argue that tetracycline derivatives, with activity against *B. burgdorferi* and HGA, should become the drug of choice for patients with confusing clinical pictures or features of more than one infection. Much of the management of patients with nonspecific presentations will depend on specific diagnostic test results. Fortunately, although babesiosis and HGA have immunosuppressive effects, these effects are not sufficient to confound the specific serologic responses to the cognate infectious agents. In the case of HGA and babesiosis, direct detection by blood smear, culture or PCR (HGA) or smear and PCR (babesiosis) are diagnostic options, but none of the direct detection approaches are very useful for the diagnosis of early Lyme disease. The diagnosis of all of these conditions may be improved by the use of next generation serologic testing and molecular diagnostic approaches, both of which are likely to make their way into increasing numbers of laboratories over the next decade.

REFERENCES


