

Laboratory aspects of common *Ixodes scapularis*-vectored Lyme coinfections: *Babesia microti*, *Bartonella henselae*, and *Anaplasma phagocytophilum*

Introduction

Over the last two decades tick-borne co-infections have been increasingly recognized as important in Lyme disease (47). Recent entomological studies have identified the deer tick endemic to the Northeastern and Upper Midwestern regions of the US, *Ixodes scapularis*, as the vector of not only *Borrelia burgdorferi* but also species of *Babesia* (36), *Bartonella* (17), *Ehrlichia* (43), *Rickettsiae* (54), and *Mycoplasma* (65) et al. A PCR amplification study of *I. scapularis* in New Jersey found prevalence of 33.6% for *B. burgdorferi*, 8.4% for *B. microti*, 1.9% for *A. phagocytophila*, and 34.5% for *Bartonella* spp. (1), though other studies have shown wide annual, seasonal and geographic variability in these rates (9, 23).

Clinically, one study showed that out of a pool of 240 Lyme cases in Rhode Island and Connecticut in the early 1990's, 11% were coinfecting with *Babesia* and suffered a longer disease course with worsened symptoms and more persistent illness (36) with malaria-like symptoms (24). *Bartonella henselae*, recently identified as a tick-borne pathogen, has been implicated as a cause of persistent encephalopathy and CNS dysfunction in coinfecting Lyme cases (17). *Anaplasma phagocytophilum*, the organism implicated in Human Granulocytic Ehrlichiosis (HGE), can lead to leukopenia, lymphopenia and thrombocytopenia (2). Up to 26% of those with proven tick-borne illness show evidence of coinfection, and in humans *Babesia* is implicated in approximately 80% of coinfection cases and HGE ranging from 3-15% (9).

Part of the problem in elucidating the exact role of coinfections in Lyme is difficulty with laboratory testing. Serological testing by IFA, EIA and Western blots for these organisms is commonly complicated by questions of what are appropriate reactivity cutoff points, band numbers or antigens, immunological responses that may or may not differ with Lyme disease coinfection either by extent or time course. Also, there are difficulties in determining species specificity (5) and in asserting what constitutes active infection versus a long-term antibody response (24, 25, 28). PCR testing can be difficult due to the often very small presence of these organisms in the blood and a lack of species specificity of amongst primers, resulting in low sensitivity that varies amongst studies (5, 35, 50). Some species do not culture well enough or quickly enough to be of diagnostic value, and blood smears can be inconsistent, subjective and difficult to reproduce (35).

***Babesia microti* detection**

Babesial parasites appear to be transmitted exclusively by *Ixodes* ticks to vertebrate hosts. An intraerythrocytic infection, the protozoa replicate in the hosts' red blood cells and are called piroplasms due to their distinct pear-shaped morphology within the erythrocyte (24). Up to 40% of nymphal ticks positive for *B. burgdorferi* are also positive for *Babesia microti* (50). Exposure to *Babesia* is common in endemic areas (44) and is often asymptomatic (69). 3-8% of blood donors may be serologically positive in endemic areas, implying a convalescent or chronic phase of infection (24, 28). While *B. divergens* was once the only form seen in Europe, recent documented cases have shown *B. microti* as well as *Babesia*-Lyme coinfection

(44). Acute babesial infection is more common in the elderly, splenectomized, otherwise immunocompromised, or those coinfecting with another tick-borne agent (24). There appears to be considerable genetic and antigenic variation in *B. microti*, with geographical clusters of different subtypes (25).

Symptoms of acute infection include malaise, chills, night sweats, myalgia, hemolytic anemia, fatigue, weight loss, hematuria, fever, nausea, and emesis et al., while laboratory signs can include elevated transaminases, alkaline phosphatases, unconjugated bilirubin, and lactic dehydrogenase, as well as thrombocytopenia and leukopenia (24). IgG antibodies prevent the entry of the protozoa into the red blood cell (24), but generally do not appear in the serum until weeks after infection while IgM antibodies appear around the time of symptomatic onset and subsequently decline (33).

There is evidence that *Babesia* can manipulate the immune response to encourage long-term parasitemia (16) and that portions of the disease may be autoimmune mediated; a study has shown that depletion of CD8+ killer T cells has no effect on or may even reduce the severity of the disease (62). NK cells have been demonstrated to play an important role in defense against *Babesia* (18) as have macrophages (45). Interestingly, the immune response to *Babesia* can induce the formation of a Th1 helper T-cell dominated environment, which has been demonstrated to increase the numbers of *Borrelia* spirochetes in coinfecting cases (49).

The most basic test for *Babesia* is a (thin or thick) blood smear with a Wright's or Giemsa stain. This test according to one study shows a sensitivity of 84%, specificity of near 100%, positive predictive value (PPV) of near 100% and negative predictive value (NPV) of 62% (35). However this method can be somewhat subjective as well as genuinely difficult to interpret (12, 24, 52), especially given that early in the course of infection as little as 1% of erythrocytes may be infected (59). The duration of detection ranges from 3 weeks to 12 weeks post-infection (58) with longer values for asplenic patients (64). A slower test involves the inoculation of hamsters with a blood sample from the patient, yet it displays low sensitivity, takes too long to diagnose the acute phase of the illness, and is costly (35).

PCR is a rapid test that can make up for some of these shortcomings, frequently using the *B. microti*-specific 18S ribosomal DNA marker (28). A group of 12 patients presenting with acute babesiosis confirmed by blood smear and IFA were all PCR positive for *Babesia microti*, and sensitivity testing showed detection almost down to the single organism per sample level (50). Another study determined for PCR testing a sensitivity of 95% and a specificity of 100%, with a PPV of 100% and an NPV of 83%, with difficulties in detection likely due to inadequate sampling, chance, the sporadic nature of the parasitemia, or sequestration in the CNS or bone marrow (35). Monitoring of the chronic convalescent state may require larger sample volumes.

Serological testing yields a complex picture as well, and is often more useful for the diagnosis of chronic infection (24). IFA testing has been demonstrated by one study across four laboratories to have ranges of sensitivity from 88-96%, specificity from 90-100%, 69-100% PPV, and 96-99% NPV (32). Cutoff values for titers can be as low as 1:64 at some laboratories (32) while values of 1:128 or 1:256 almost completely eliminate false positive results (24). Titers can remain above normal for 13 months to 6 years after infection (49) and IgG declines less rapidly in cases of persistent infection compared to those who clear their infections in less than 3 months (24).

IgM IFA testing is more problematic given its tendency to generate false positive results (29). Cross-reactivity with other protozoal parasites may be responsible for this (24),

and it has been shown that patients with systemic lupus erythematosus and rheumatoid arthritis can generate false positives, albeit through different mechanisms (49). One test developed for the diagnosis of acute babesiosis via IgM showed a sensitivity of 91%, a specificity of 99%, a PPV of 86%, and an NPV of 99% with a titer cutoff of 1:32, implying that this could be a useful if incomplete test for acute babesiosis (33).

The search for fast, affordable, and more accurate tests for acute babesiosis led to the development of an immunoblot serological test that detected 9 *B. microti* specific bands. In a trial of more than 100 people, all the cases of babesiosis showed at least one band (all were IFA-positive as well). 4% of the subjects who had no signs of babesiosis, including a significant number of people with other tick-borne illness, demonstrated bands but none were IFA-positive. If 2 bands were set as a threshold for the test, sensitivity was 96%, specificity 99%, PPV 96%, and NPV 96% (59).

In screening for transfusion purposes, a test was developed to pick up infection in the chronic/subclinical state whose titers and PCR data are unreliable. Identifying and mapping two epitopes, BMN1-17 and MN-10, specific to 2 serocomplementary *B. microti* antigens, an EIA was developed that detected 98 out of 107 sera that were IgG blot positive, and in those 9 nonreactive cases and 4 equivocal ones the patients had low IgG titers indicative of past exposure (28). Subsequent IFA testing revealed 60 'false-positives,' which could mean the test is less useful for detecting recent infection but is effective for screening for exposure. Promising for the future is a study reporting the detection of shed antigens that can be detected via ELISA at the subclinical level of infection (26).

***Bartonella henselae* detection**

The genus *Bartonella* is composed of short, fastidious gram-negative rods (31) that cause extracellular as well as chronic intracellular erythrocytic or endothelial infection of vertebrate hosts without hemolysis, via an arthropod vector (5, 30, 57). The domestic cat is the characteristic host of *Bartonella* (55). *Bartonella* species can cause chronic asymptomatic bacteremia in mammalian hosts (5), though humans are only incidental hosts when immunocompetent (30). The immunology of the infection is complicated, however; there is wide variation in the time courses of IgM and IgG titers between acute and chronic cases (19). *Bartonella quintana* is established as the cause of both trench fever and many cases of endocarditis (30) and is closely related to *B. henselae* (38). *B. henselae* was identified in 1992 as the agent of Cat-Scratch Disease or CSD (5), which normally is locally restricted, manifests as regional lymphadenopathy and resolves without treatment (19, 30, 55).

However, it can penetrate the CNS and lead to neurological complications in as many as 11% of cases (51), including encephalopathy (39), cranial nerve palsy and peripheral nerve involvement (66), new-onset epilepsy (46) and neuroretinitis (13). These complications of CSD are particularly common in children or young adults among the immunocompetent (Carithers-1200). More extreme forms of illness involve prolonged fever, hepatic and splenic abscesses, vertebral osteomyelitis, Parinaud's oculoglandular syndrome, optic neuritis, autoinflammatory meningoencephalitis, or osteolytic bone lesions (5, 30, 55, 61). In the immunosuppressed, *Bartonella* bacteremia has been proposed as a possible cause of dementia and neuropsychiatric deterioration in HIV-positive patients that are IgM positive for *B. henselae* (88Jacom), as well as aseptic meningitis (39).

B. henselae has been indicated as the cause of bacillary angiomatosis and peliosis hepatitis (30). Exposure of THP-1 macrophages to *B. henselae* has demonstrated a paracrine signaling loop that is sufficient to cause via adherence or penetration by the bacteria the

modulation of VEGF and interleukin-1 β production, resulting in endothelial cell proliferation (53). *B. henselae* may cause up to 3% of all cases of endocarditis (30 Fournier), especially in the immunocompromised (31). *B. henselae* is remarkable for its worldwide distribution (30). It has only recently been identified as a tick-borne disease that can present as a co-infection with *B. burgdorferi* without hallmark signs of CSD, potentially causing persistent encephalopathy, neurological symptoms and neuropsychiatric dysfunction even with successful treatment for *B. burgdorferi* (17).

Complicating the picture of illness is the difficulty of obtaining a definitive laboratory diagnosis. Detection of *B. henselae* from the blood is unlikely in the absence of systemic disease (5). Histopathologic examination is of little clinical interest but was important in the initial identification of *B. henselae*, given that it stains well by the Warthin-Starry method (99, 110 Anderson). Culture is difficult; until 2002 there were only 14 reported isolates from cases of CSD (19). Culture requires extensive incubation and may be extremely insensitive in cases that are not highly bacteremic (5), including those that have received treatment. One recent group developed a culture medium containing hemin that subsequently grew cultures from 20.5% of PCR-positive specimens for CSD in the study (19).

PCR was important in the initial discovery and description of *B. henselae* (5). The initial tests were based on the highly conserved 16s rRNA sequence identified from bacillary angiomatosis patients (160 Anderson), though these promoters are broadly reactive across many families of bacteria and are 97.6 to 99% identical amongst *B. spp.* (3 Liang). This has led to concerns about contamination and reduced specificity (5). This question is often addressed by RFLP analysis and sequencing of the DNA products to isolate *B. henselae* specific product. One study of lymph node biopsy-PCR for CSD showed a sensitivity of 76% and a specificity of 100%, with those values and the positive predictive values rising with restrictions of the control group to eliminate definite CSD-negative cases (21).

Generally, PCR displays excellent specificity, and sensitivity that varies directly with the accumulation of supportive clinical or laboratory data. The sensitivity of PCR is often well below 80%, however (21) in non-bacteremic cases. One additional cause of the low sensitivity may also be the inactivation by Ig of many DNA polymerases (44 Fournier). A single-step PCR assay was developed by Jensen et al. that eliminated the need for RFLP analysis or sequencing to differentiate between bacterial species by producing a promoter that produced a different length product for each *Bartonella* species, expediting test results and reducing the risk of contamination. It detected *B. henselae* in the blood of 100% of samples with 50 to 100 CFU/ml, 85% with 30 CFU/ml, and 75% with 10-20 CFU/ml (31).

Serological testing for *Bartonella* often displays inadequate sensitivity and specificity (21). Cross-reactivity amongst *Bartonella* species and with *Chlamydia psittaci* has been well documented (95 Anderson), though recently developed EIA and IFA assays are showing increased specificity (5). The sensitivity of IgG IFA has been calculated in a study by Bergmans et al. for probable CSD cases as 40.9%, with IgM IFA sensitivity at 50%. EIA sensitivities were 9.5% for IgG and 71.4% for IgM, with PCR sensitivity of 86.4% for comparison, with titers designed to ensure at least 95% specificity for all tests. The study also revealed widely disparate IgG and IgM antibody kinetics amongst IgM positive CSD patients, and variable concordances between serological testing and PCR results. These low sensitivities may be due to the high prevalence of IgG antibodies in the population at large and particularly from family members of the patients (10).

Another study by Maurin et al. evaluated a Focus Technologies test for IgG IFAs, obtaining a sensitivity of 91.2% and specificity of 87% for IgG in CSD patients with a titer cutoff of 64, with a cutoff of 128 improving specificity but reducing sensitivity to 85%. The test only reported IgG titers greater than 1024 for bacteremic endocarditis in 16.7% of cases, a figure that a test developed by the researchers themselves would considerably improve on (75% by their own assay) (42). Western blot analysis of *Bartonella* has shown promising results, with one study successfully differentiating between nine different *Bartonella* species via distinct protein profiles produced by SDS-PAGE digestion (38).

***Anaplasma phagocytophilum* detection**

Anaplasma Phagocytophilum, once part of the genus *Ehrlichiae* and closely related to its many strains infecting humans and animals, is a gram-negative, obligatory intracellular bacterium that targets granulocytic leukocytes (40 or 41?). Its primary vector is the *Ixodes scapularis* tick, with the *Peromyscus leucopus* mouse a possible reservoir (Courtney). Successfully identified as the causative agent of Human Granulocytic Ehrlichiosis by a nested PCR assay in 1993 (14), the first cases clustered around the area of the Upper Midwestern United States (Bakken-A new species emerging), but it has been shown to be prevalent in the Northeastern US as well as on the American west coast and in Europe (40 or 41?). A study in Westchester County, New York recorded as seropositive by IFA 11.3% of blood donors, 5.1% of Lyme-negative controls, and 21.4% of erythema migrans positive Lyme cases for *A. phagocytophilum* (3).

At present, HGE is believed to be the second-most common tick-borne infection in the US after *B. burgdorferi* (40). A potentially fatal febrile syndrome, common symptoms of acute HGE include myalgia, headache, thrombocytopenia, leukopenia, and elevated serum aminotransferase levels (Bakken-14HGE). Severe manifestations include septic or toxic-shock-like syndromes, coagulopathy, myocarditis, demyelinating polyneuropathy, rhabdomyolysis, acute respiratory distress syndrome, hemorrhaging, and opportunistic infections (15). There is disagreement about whether HGE can cause a rash (15, Bakken014). Most infections are self-limiting and subclinical, but up to 56% of acute cases require hospitalization and as many as 1% can be fatal (8,15). The infection and antibody response can be persistent even after treatment (63).

Blood smears of confirmed HGE cases have demonstrated the invasion of as many as 42% of host neutrophils (8) in severe cases. Replicating intracellularly, it has been demonstrated that *A. phagocytophilum* can reduce that rates of neutrophil apoptosis in vivo (60) and therefore increase the lifespan of its immediate host. In terms of antibody kinetics, serology for HGE is usually negative in the first week of acute infection (40), problematic given the dangers of the acute infectious syndrome. IgM titers, appearing after one week, are shown by one study to disappear no later than 41 days post infection (67), while IgG titers increase over time and peak weeks to months after the acute phase of infection (70), with a subsequent long-term decline (bk). Intriguingly, a mouse study has demonstrated that coinfection with *B. burgdorferi* can lead to a reduced antibody response to *A. phagocytophilum* and an increase in the amount of *B. burgdorferi* DNA amplified by PCR (22).

Definitive diagnosis of HGE can be established by peripheral blood smear (67). Smears can reveal distinctive, structurally complex, membrane-bound bacterial aggregates (43) in differing numbers of granulocytes, often appearing under Wright's stain an intense blue darker than nearby chromatin (6). However, the sensitivity of this method is highly variable and can be dependent on timing of the sample and the skill of the examiner (6). Culturing the

organism is difficult and takes enough time to be of use only for confirmation of illness after the fact (27). The original identification of the organism was made by PCR after attempts at culture failed (14).

PCR is a valuable tool for HGE diagnosis because it can provide positive results during the acute phase of infection (15). PCR tends to be positive in acute, highly bacteremic cases (Massung/Slater 97) and can become negative after the acute phase ends or after 3 days of treatment with doxycycline (15). PCR sensitivity varies as well with the number of fulfilled clinical criteria, and there can be PCR-negative HGE cases throughout the course of illness (8). A comparative study of culture-positive and culture-negative cases showed that culture-positive cases, which tend to be acute, were 100% positive by PCR, and only 20% positive in culture-negative cases (27). A major problem is specificity, as many PCR assays commonly replicate DNA from other *Ehrlichia* species as well as *R. rickettsii*, *B. henselae*, and others, especially as many PCR assays are based on the highly conserved 16S rRNA region that is highly conserved in bacterial species (40). The same study did show, however, distinct banding patterns for each species via immunoblot analysis.

Though many studies have shown high PCR sensitivities and specificities experimentally only to have reports of poor performance clinically (27), improved versions of assays such as the GE9f/GE10r *A. phagocytophilum*-specific primers have shown sensitivity as high as 86% and specificity of 100% (Dumler/Edelman). Other studies have shown sensitivities for the same primer as low as 43% (Bakken/Dumler). Other targets for polymerization include *rrs*, the *groESL* operon, *ankA*, *msp2*, and *epank1*, a highly specific primer that displayed 100% sensitivity among seropositive patients (68). There are also disparities amongst laboratories and PCR kits that may be clinically significant (40).

Serological diagnosis can be problematic as well, especially given that the tests are often negative during acute illness (Massung/Slater 98). An IFA is the most common test for confirming a diagnosis of HGE (Bakken/Dumler 98), though some groups claim superior results with ELISAs (32). A four-fold elevation in the IFA is considered positive for *A. phagocytophilum* (6) and titers of 1:80 are considered positive as well (8), though other groups have used lower cutoffs to increase sensitivity (37). In one study, ROC analysis determined the 1:80 cutoff titer to have the best balance of sensitivity and specificity, with sensitivities ranging from 82-100% and specificities from 67-100%. The same study showed convalescent a IgM SE value of only 30% and SPC of 79% compared to 88.5 and 93%, respectively, for IgG. In the acute stage of illness, IgM SE was 33% and SPC 83%, with respective values for IgG of 44% and 100%, consistent with what is known about the antibody kinetics of HGE (67). Another study showed that the antibody response becomes apparent on average at 11.5 days, and that 91.5% of definite cases will seroconvert during the course of illness and recover (4).

False-positive results are not uncommon (15), likely due to the cross-reactivity of IFA assays with *Bartonella* (11), *E. equi* (8) and other *Ehrlichia* and *Rickettsii* species. Consequently, there have been many attempts to find *A. phagocytophilum*-specific antigen that could be used in less ambiguous immunoblot testing (48). The P44 gene, a 44-kDa outer membrane protein of *A. ph.*, is an immunodominant agent, but has been shown to have multiple polymorphic variants via Southern blot analysis (71). Another study showed distinctive banding patterns in HGE isolates indicating a strain pleomorphism in the major antigenic proteins of *A. phagocytophilum* (Zhi/Rikihisa). An evaluation of a two-tiered testing system with IFA/EIA followed by confirmatory WB showed the EIA to be superior to IFA in detecting Western blot-confirmed antibodies to the infection, while revealing on the WB a reproducible

blotting pattern stressing the importance of the 42-45kDa antigen as a key marker of *A. phagocytophilum* infection (Ravyn).

Conclusion

The emergence of tick-borne infection has become a major public-health issue in the United States and abroad, particularly as improved diagnosis reveals larger prevalence for such illnesses. The *Ixodes scapularis* tick has been the primary vector in the northeastern and Midwestern US and is the best characterized host. The response to this emerging threat is complicated by the inconclusive nature of much of the diagnostic laboratory testing for the diseases, considering their often-vague symptoms. While *B. burgdorferi* infection is the best-known and characterized of the tick-borne infections, the numerous coinfecting agents have been demonstrated to cause significant complications of their own as well as functioning as modulatory agents that may complicate the course of 'Lyme disease.'

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