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STUDIES TO IMPROVE THE ABILITY OF CULTURE MEDIUM TO RECOVER *BORRELIA MIYAMOTOI*

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STUDIES TO IMPROVE THE ABILITY OF CULTURE MEDIUM TO RECOVER

BORRELIA MIYAMOTOI

By Kaitlyn Craun

We recommend acceptance of this thesis in partial fulfillment of the candidate's requirements for the degree of Master of Science Microbiology: Clinical Microbiology

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ABSTRACT

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The relapsing fever spirochete *Borrelia miyamotoi* was recently detected in hard-bodied *Ixodes scapularis* ticks that also transmit Lyme disease-causing *B. burgdorferi.* As a result, methods of laboratory diagnosis that rely on detecting specific antibody responses are likely confounded as the spirochetes share similar proteins. Efforts to better understand this possibility have been hampered due to the absence of a medium capable of recovering *B. miyamotoi* from a human who contracted the illness in the United States. In this study, MKP-F culture medium typically used to cultivate a laboratory-acclimated *B. miyamotoi* isolate was significantly improved by eliminating gelatin and adding 10% human serum (MKP-H). However, the increased efficacy was not sufficient to recover the spirochetes from a patient who contracted the spirochetes. In a preliminary experiment, it was also confirmed that patients infected with *B. miyamotoi* produce antibodies that react commonly with multiple *B. burgdorferi* proteins, including the C6 peptide thought to be highly specific for Lyme disease spirochetes, which highlights the necessity of continued effort to improve the efficacy of laboratory culture medium sufficient to recover *B. miyamotoi* spirochetes from local patients.

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BACKGROUND

General information. Ticks, hematophagous arthropods, were considered benign pests for centuries. However, a tick was identified in 1893 (1) as the vector responsible for transmitting *Babesia bigemina,* the causative agent of cattle fever, and numerous other viral, bacterial, and protozoan pathogens that infect humans via transmission from infected ticks have since been described. To date, at least 200 species of ticks that feed on humans have been identified, and most transmit disease-causing pathogens.

Ticks can be divided into two major families consisting of either soft-bodied or hard-bodied ticks. The families are distinguished primarily by either a scutum or hardened shield that covers the dorsal anterior portion of females or the entire body of males (Ixodidae), respectively, or a leathery cuticle (Argasidae) (2). In addition, Argasidae soft-bodied ticks acquire blood meals only intermittently, while Ixodidae hard-bodied ticks feed much more frequently. The hard-bodied ticks also have more developed mouth parts that anchor to the host and release a cement-like substance. This in turn establishes a feeding channel that also serves as a conduit for migration of microorganisms from the tick to the mammalian host.

Within the Ixodidae family, ticks of the genus *Ixodes* are extremely significant because each member transmits human pathogens. For example, *I. ricinus* is a primary vector for a single-stranded *Flaviviridae* RNA virus that causes tick-borne encephalitis

(2). The illness initially presents as mild fever or headache that can quickly progress to encephalitis or meningitis that can be fatal (2). In addition, *I. holocyclus* causes Queensland tick typhus via transmission of the protozoan *Rickettsia australis* (3). The illness normally manifests as fever, headache, and myalgia, but there have been instances of severe abnormalities that have included gangrene of the extremities or respiratory failure (3). Furthermore, *I. pacificus* ticks, which are found in the western United States (US), transmit *Ehrlichia chaffeensis*, the causative agent of human monocytic ehrlichiosis (4). The organisms most often cause relatively mild symptoms that include low grade fever and headache, but multi-organ failure is also a possibility (5).

***Ixodes scapularis*.** The most common *Ixodes* spp. tick in the US is *I. scapularis*, which is correctly classified as the black-legged tick, but is often referred to as the deer tick. The deer tick was originally classified as *I. dammini*, but Oliver et al. (6) showed by more critical genetic analyses that *I. scapularis* was the more appropriate classification. Deer ticks commonly feed on at least three hosts over an approximately 2-year time period. The life cycle begins when an engorged adult female deposits up to 2000 eggs in a single cluster during the spring. The eggs then yield larvae that most often acquire a blood meal after attaching to a small rodent or bird. The blood meal in turn stimulates the larva to molt into a nymphal tick that must also obtain a blood meal, preferably from a rodent, and the blood in turn stimulates molting into a male or female adult. The female adult subsequently attaches to a larger vertebrate, most often a white-tailed deer, where the tick awaits impregnation by an adult male. After impregnation, the female completes the final blood meal that then provides the impetus for the formation and deposition of eggs.

Interestingly, deer ticks were present in only limited regions within the eastern US at the turn of the century, primarily because the preferred white-tailed deer host was extremely rare due to predation, widespread deforestation, and lack of harvest regulations (7). However, more rigid logging and hunting laws were initiated during the 1930s, and the result was the rapid rebound of both the white-tailed deer and deer tick populations. Since then, white-tailed deer and the deer ticks that prefer them as terminal hosts have expanded into most regions of the US (8).

Disease transmission by *I. scapularis*. Similar to other *Ixodes* spp., *I. scapularis* ticks are also efficient vectors of human illness, primarily because they obtain blood from multiple mammalian hosts that are reservoirs for a wide variety of pathogens and inject large volumes of tick saliva that often contains the pathogens as they are ingesting blood (1, 9). In addition, there is ample time for transmission as the larval and nymphal ticks remain attached for up to 8 days, and the adults feed for up to 12 days (1).

The most well-known illness caused by *I. scapularis* ticks is Lyme disease from transmission of spirochetal *Borrelia* spp. bacteria. *B. burgdorferi* sensu stricto (ss) is the most common causative agent of Lyme disease in the US. The illness is characterized by the development of a "bulls-eye" erythema migrans rash that may appear coincident with constitutional abnormalities such as headache, fatigue, arthralgia, and myalgia. Untreated infections can then progress to cause more serious disorders of the joints, heart, and nervous system (10). The most common late manifestation in US patients is swelling of the large joints, most likely because *B. burgdorferi* ss have a predilection for joint tissue (11).

Even before the emergence of Lyme disease, deer ticks were widely recognized vectors of other human bacterial and viral pathogens. Predominant among other pathogenic bacteria are *Babesia microti*, the causative agent of babesiosis (12), and *Anaplasma phagocytophilum,* the causative agent of anaplasmosis (13). Researchers also showed recently that deer ticks can transmit Powassan virus, a *Flaviviridae* thought previously to be transmitted only by *I. cookie* (14). This is especially worrisome because Powassan virus can cause severe neuroinvasive illness characterized by encephalitis or meningitis, and the fatality rate approaches 10% if this occurs. In addition, greater than 50% of individuals who contract the viral illness experience severe neurological abnormalities for months to years after they are no longer infected (14, 15).

Emergence of *B. miyamotoi*. There is no doubt that deer ticks are efficient vectors of human pathogens, so it is not surprising that researchers continue to investigate the ability of the parasites to transmit additional microorganisms. Most recently, researchers examined serum samples from patients with unexplained fevers who resided in Rhode Island, Massachusetts, New England, and New York and found that multiple samples contained antibodies that bound *B. miyamotoi* proteins, a *Borrelia* spp. categorized as a causative agent of human relapsing fever (16). *B. miyamotoi* was first isolated from *I. persulcatus* ticks collected, in Japan (17) and the organisms were initially considered non-pathogenic (17). However, the spirochetes were subsequently confirmed as the causative agent of relapsing fever in several Russian patients (18) and, since this discovery, *B. miyamotoi* has been detected in several species of *Ixodes* ticks including *I. scapularis* (19). In addition, Barbour et al. (19) subsequently showed that *I. scapularis* ticks captured from 15 states in the northeastern US harbored the spirochetes.

These findings were especially noteworthy because, previous to these reports, *Borrelia* spp. that caused relapsing fever were considered associated exclusively with soft-bodied ticks or lice (20, 21). In fact, Lyme disease-causing *Borrelia* spp. were considered exclusive to *Ixodes* spp. ticks and relapsing fever-causing spirochetes were associated only with soft-bodied ticks (20, 21). More recently, however, Jobe et al. (22) detected *B. miyamotoi* spirochetes in *I. scapularis* ticks collected from areas near La Crosse, WI and the researchers also confirmed that local individuals had developed relapsing fever from infection with the organisms.

Characteristics of relapsing fever. Patients with relapsing fever typically have a flu-like illness characterized by episodes of fever that wax and wane every few days (23). The recurrent fevers occur because the *Borrelia* spp. regulate the expression of surface-expressed proteins. The most likely mechanism is differential expression of variable large (Vlp) and small proteins (Vsp) similar to those utilized by *B. hermsii* (24, 25) since recent studies (25, 26) have confirmed *B. miyamotoi* possess similar genes. In the case of an infection with *B. hermsii*, the spirochetes initially express Vlp, which induces the production of anti-Vlp antibodies as the organisms are killed and processed by host phagocytic cells. The immune pressure from the antibody response then causes the spirochetes to decrease the expression of Vlp and increase the expression of Vsp. This allows some organisms to escape elimination and subsequently proliferate, and the result is the fever waxes and wanes as the spirochete load is reduced and then rebounds.

Characteristics of illness from *B. miyamotoi*. Similar to other pathogens that cause relapsing fevers, infection with *B. miyamotoi* can manifest as a flu-like illness characterized by recurrent fever episodes (18, 27), but the characteristic fever may not

occur if the patients are treated effectively soon after the organisms have colonized the individual (18). However, the clinical syndrome is documented (28) when the patient is not initially treated appropriately. In the most severe cases, relapsing fever spirochetes can also cause meningoencephalitis, especially if the infected individual is immunocompromised (29–31).

Treatment of relapsing fever. There is currently no formal recommendation for treating illness caused by *B. miyamotoi*. However, researchers (32, 33) have confirmed the spirochetes are susceptible to doxycycline, and successful treatments by using this antibiotic have been documented (32, 34). This is especially significant, because the treatment doxycycline is also commonly used to effectively eliminate Lyme disease spirochetes. However, researchers (32, 33) also showed recently that the spirochetes could resist elimination by treatment with amoxicillin, which is also a common treatment for Lyme disease, so studies to more thoroughly evaluate antibiotic resistance remain necessary. In addition, as is common with infections caused by spirochetal bacteria, effective antibiotic therapy often causes a Jarisch-Herxheimer reaction that manifests as hypotension, tachycardia, chills, rigors, and elevated body temperature from the large amounts of cytokines that are released as the spirochetes are killed (35).

Direct laboratory confirmation of relapsing fever. Historically, confirming infection by directly detecting the spirochetes was most often accomplished by visualizing the organisms in blood smears (36). However, the procedure suffers from lack of sensitivity, especially if only a few organisms are present, and subjectivity can be problematic when discriminating artifacts from spirochetes. As a consequence, researchers have developed polymerase chain reaction (PCR) tests that target *B.*

miyamotoi-specific regions of several genes including primarily the glpQ (22) or flaB flagellin genes (22, 37). The PCR tests have greatly increased the accuracy of laboratory detection, especially during early infection, but the tests are not widely-available (34, 38).

Indirect laboratory confirmation of relapsing fever. The most common laboratory method for confirming relapsing fever remains detecting relevant antibody responses. However, antibody tests that utilize the whole organism or multiple proteins can be confounded significantly by non-specific binding of antibodies formed against unrelated antigens, especially antibodies induced by infection with closely-related organisms such as the *Borrelia* spp. that cause Lyme disease (27, 36). Conversely, reports (27, 36) have demonstrated the ability of antibodies from *B. miyamotoi*-infected patients to also bind *B. burgdorferi* antigens and, therefore, confound accurate confirmation of Lyme disease. This cross-reactivity is especially problematic in regions of the US where cases of relapsing fever or Lyme disease are common.

The current most reliable test for discriminating infection with relapsing fever spirochetes is to detect only antibodies that bind the glycerophosphoryl diester phosphodiesterase (GlpQ) protein, since expression of this protein is unique to relapsing fever-causing spirochetes (36, 38). However, the test apparently suffers from lack of sensitivity during early infection, since Molloy et al. (34) recently showed that only 11% of the immune sera collected from patients with early cases of *B. miyamotoi* infection contained antibodies that bound GlpQ. Therefore, studies to identify additional specific antibody responses are ongoing. One recently-described (26) possibility is to detect antibodies that bind the variable membrane proteins (VMP). However, the VMP genes are extremely heterogeneous (26), so it will be difficult to design studies to accurately

identify specific antibody responses that could be universally-produced by patients with the illness.

STUDY RATIONALE AND RESEARCH PLAN

The recent discovery that *I. scapularis* ticks can also transmit *B. miyamotoi* magnifies the importance of studies to gain a greater understanding of this new finding. Especially critical are efforts to elucidate specific antibody responses that occur during human infection, since characterization can lead to development of more effective diagnostic tests or vaccines. An important shortcoming is that relapsing fever and Lyme disease spirochetes share many of the same proteins (27), so there are currently no widely-available laboratory antibody-based tests that can reliably discriminate infection with either pathogen. Therefore, studies that discriminate antibody responses specific to infection with *B. miyamotoi* are needed. However, a likely critical component of this effort will be evaluating the antibody responses using an organism recovered from an infected human. This may be essential, because the antigenicity of spirochetes recovered from laboratory culture medium or mammalian hosts has historically diverged significantly, which has in turn caused considerable confusion and even misdiagnosis. For example, the organisms express high levels of OspC and expression of OspA is minimal when the spirochetes are colonizing a mammalian host (39, 40), and the result is the production of OspC antibodies predominates during early infection. However, Lyme disease spirochetes that are cultured in the laboratory express large amounts of outer surface protein (Osp) A and little or no OspC within one generation time after being inoculated into laboratory culture medium (39), so early studies that relied on laboratory cultured spirochetes to develop clinical tests were often erroneously based on the

supposition that high levels of anti-OspA antibodies should be produced by Lyme disease patients when, in fact, OspA antibodies are seldom produced during the illness.

The variable protein expression may therefore also be problematic when characterizing the antibody responses that occur during illness caused by infection with *B. miyamotoi*, and it is likely that accuracy may be dependent on experimentation that utilizes an isolate recovered from a human patient. However, efforts to recover the spirochetes from a human patient from the US have been unsuccessful to date. This is especially puzzling since researchers (41–44) have successfully recovered *B. miyamotoi* from infected *I. scapularis* ticks and even human patients from Russia by using a modified Kelly-Pettenkofer medium in combination with fetal calf serum (MKP-F). Therefore, the MKP-F apparently lacks one or more key ingredients that are critical for the survival of the *B. miyamotoi* spirochetes infecting US patients, which also highlights the possibility of important antigenic differences among the organisms. In fact, researchers now routinely characterize the relapsing fever spirochetes that infect US patients as *B. miyamotoi* sensu lato to signify the unknown antigenic differences compared to the cultivable *B. miyamotoi,* designated sensu stricto because they have been characterized genetically, that have been successfully recovered from Russian and Japanese patients (17, 45).

In this study, therefore, I propose to use *B. miyamotoi* HT31, an isolate recovered from a tick captured in Japan (17) that grows reliably in MKP-F medium, to evaluate whether varying the quality and amounts of specific ingredients in the culture medium will increase the efficacy sufficient to recover *B. miyamotoi* from an infected human blood sample. To increase my chances of success, I will also develop procedures to

minimize the impact of exposing the spirochetes in human blood to ethylenediaminetetraacetic acid (EDTA), because the ingredient can be lethal to microorganisms and human blood is routinely collected in vacutainer tubes that contain EDTA to prevent clotting. In addition, I will screen blood samples from patients who present with suspected tick-borne illnesses for infection with *B. miyamotoi* by using the PCR test offered routinely in the Gundersen Health System Microbiology research lab that simultaneously detects *B. miyamotoi, Babesia microti,* or *A. phagocytophilum* in blood samples to ensure that I have a readily-available supply of appropriate culture material.

If my effort results in the successful recovery of a *B. miyamotoi* isolate, I then propose to use the organisms to characterize the IgM and IgG antibody responses in immune sera from *B. miyamotoi*-infected patients and also perform preliminary studies to evaluate the ability of the antibodies to bind antigens important for discriminating infection with *B. burgdorferi*. Accomplishing these objectives will yield valuable information that should be useful for future studies, especially those designed to develop accurate laboratory test procedures.

MATERIALS AND METHODS

Organisms. *B. burgdorferi* ss 297 was originally recovered from human spinal fluid (46). The spirochetes were grown at 34°C in Barbour-Stoenner-Kelly (BSK) medium to a concentration of approximately 5 x 10^7 organisms per milliliter, aliquoted in 500 µl-amounts into individual sterile 1.5 ml screw cap tubes (Sarstedt, Newton, NC), and stored at -80°C until used.

B. miyamotoi ss HT31, recovered from an *I. persulcatus* tick captured in Japan (17), was kindly provided by Dr. Martin Schriefer (Centers for Disease Control and Prevention). The spirochetes were cultured at 34°C in MKP-F medium and, after reaching a concentration of $\geq 10^6$ organisms per milliliter, the spirochetes were aliquoted as described and stored at -80°C until used.

Laboratory culture media. *B. burgdorferi* ss 297 were grown in BSK medium that was prepared as previously described (47, 48). Specifically, 6 g of *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer (Sigma-Aldrich Co., St. Louis, Mo.), 5 g of neopeptone, 2.5 g of TC yeastolate (Becton, Dickinson and Company, Sparks, MD), 0.7 g of sodium citrate, 5 g of glucose, 2.2 g of sodium bicarbonate, 0.8 g of sodium pyruvate, 0.4 g of *N*-acetyl-D-glucososamine (Sigma-Aldrich), and 50 g of fatty acid-free bovine serum albumin fraction V (Boval Company, L.P., Cleburne, TX) that had been purified by using organic solvents were added to a 2-liter flask that contained 900 ml of distilled water. The suspension was slowly stirred until the ingredients were dissolved, and the pH was then adjusted to 7.5 by using 5N NaOH. In

addition, 14 g of gelatin was added to a separate flask that contained 200 ml of distilled water, and the suspension was autoclaved at 121˚C for 15 minutes. After autoclaving, the gelatin suspension was cooled to 56˚C, and 6.33 g of 10X Connaught Medical Research Laboratories (CMRL) 1066 liquid medium without glutamine (United States Biological, Salem, MA) and 64 ml of heat-inactivated rabbit serum (Life Technologies, Carlsbad, CA) (56˚C, 45min) were added prior to combining the two suspensions. Finally, a positive pressure pump was used to force the medium through a manifold that contained stacked 0.80 µm, 0.45 µm, and 0.22 µm filters, and the sterilized BSK was stored at 4°C until used.

B. miyamotoi ss HT31 was cultured in modified Kelly-Pettenkofer medium (MKP-F) that was also prepared as previously described (41). Specifically, 3.9 g of *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid buffer, 3.3 g of glucose (Sigma-Aldrich), 2 g of neopeptone (Becton, Dickinson), 1.4 g of sodium bicarbonate, 0.52 g of sodium pyruvate, 0.46 g of sodium citrate, 0.26 g of *N*-acetyl-D-glucososamine (Sigma-Aldrich), and 32.8 g of bovine serum albumin (BSA) fraction V (Boval) obtained by fractionation with organic solvents were combined in 730 ml of distilled water. In some instances, the BSA fraction V was replaced with product from another manufacturer (Sigma-Aldrich) that had been either fractionated with organic solvents, cold ethanol, or heatshock.

The mixture was then mixed slowly until the ingredients were dissolved, and the pH was adjusted to 7.5 by using 5N NaOH. In addition, 14 g of gelatin (Becton, Dickinson) was added to a separate flask that contained 200 ml of distilled water prior to autoclaving the suspension at 121°C for 15 minutes. The autoclaved suspension was then

cooled to 56°C prior to adding 6.33 g of 10X CMRL 1066 medium without glutamine (US Biological), 44.8 ml of rabbit serum heat-inactivated by incubation in a 56°C water bath (Life Technologies) for 45 min and 100 ml of heat-inactivated (as described) fetal bovine serum (HyClone Laboratories, Logan, UT). Finally, the mixtures were combined and sterilized by positive pressure passage through a 0.22 µl filter as described above. The MKP-F medium was aliquoted into sterile 50 ml Falcon tubes (Fisher Scientific, Pittsburgh, PA) and stored at 4˚C until used. In some instances, the MKP-F medium was modified by omitting the gelatin and adding a 10% volume of human serum (Sigma). The modified MKP-F medium was then designated MKP-H.

Normal human blood. Blood samples from a volunteer donor without history of tick bite or tick-borne illness was collected by a phlebotomist in the UW-L Health Science Center. The blood was collected in 6 ml purple cap (K_2-EDTA) sterile vacutainer tubes and used immediately.

PCR detection of *B. miyamotoi*. Blood samples that contained *B. miyamotoi* DNA were detected by PCR. A one ml-volume of blood was centrifuged at 2,500 x g for 10 minutes. Following centrifugation, 200 µl of the intermediate cell layer (buffy coat) was transferred to a 1.5 ml microcentrifuge tube, and the DNA was extracted by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Briefly, 20 µl of QIAGEN protease, 7 µl of DNA from a lambda bacteriophage carrying a 4 kb region of mouse hepatitis virus (DNA extraction control), and 200 µl of buffer AL were added to the cell suspension prior to pulse-vortexing and then incubating for 10 minutes at 56°C. Following incubation, 200 µl of 100% ethanol was added, and the sample was transferred to a QIAamp spin column contained within a two ml collection tube. The spin column

was centrifuged for one minute at 6,000 x g, 500 µl of Buffer AW1 was added, and the buffer was removed by centrifuging again at 6,000 x g for one minute. The spin column was then placed in a clean collection tube and 500 µl of Buffer AW2 was added prior to centrifugation for 3 minutes at 20,000 x g. Finally, 50 µl of AE buffer was added, and the column was incubated at room temperature for 5 minutes. After incubation, the spin column was centrifuged for one minute at 20,000 x g, and the eluted DNA was transferred to a sterile 1.5 ml centrifuge tube.

B. miyamotoi DNA was detected by combining 5 µl of the extracted DNA with 19 µl of a mastermix that contained 12.5 µl of A buffer (Life Technologies), 4.5 µl of TP primer/probe mix, 2.5 µl of EraMix suspension (EraGen Biosciences, Inc., Madison, WI) and 0.5 µl of AmpliTaq Gold DNA polymerase in a 0.2 ml PCR tube (Life Technologies). The primer/probe mix consisted of primers and probes specific for the *msp*2 gene of *Anaplasma phagocytophilum*, the 18S rRNA gene of *Babesia microti*, and the *GlpQ* gene of *B. miyamotoi* (Eurofins Genomics LLC, Louisville, KY) (Table 1). The DNA was then amplified by an initial denaturation/ enzyme activation cycle at 95°C for one cycle, 40 cycles of denaturation at 95°C, a separate cycle of annealing at 58°C, and a final extension cycle at 72°C. The amplified products were detected by measuring the fluorescence intensity of the individual probes (Stratagene Mx 3000P; Agilent Technologies, Santa Clara, CA). Each run contained samples with DNA from each organism as positive controls, a negative buffer control, and an internal control of unrelated DNA.

Microscopic detection of *Borrelia miyamotoi*. Blood samples that contained *B. miyamotoi* DNA were also examined for motile spirochetes. The blood sample was

TABLE 1 Primers and probes used by the Microbiology Research Laboratory at Gundersen Health System for detecting tick borne infections by PCR.

Organism	Primer/ Probe	Sequence
Anaplasma phagocytophilum	Forward	5'-ATGGAAGGTAGTGTTGGTTATGGTATT-3'
	Reverse	5'-TTGGTCTTGAAGCGCTCGTA-3'
	Probe-FAM[a]	5'FAM-TGGTGCCAGGGTTGAGCTTGAGATTG-BHQ1-3'
Borrelia miyamotoi	Forward	5'-GCACAATTATTTCCCAATC-3'
	Reverse	5'-TCAGGATCAAATCTTTCAC-3'
	Probe-TEX[b]	5'-TxRed-AACGGAAGATATTACGCTACTGACT-BHQ2a-3'
Babesia microti	Forward	5'-TCGCGTGGCGTTTATTAGAC-3'
	Reverse	5'-CCGGCAAAGCCATGCGATT-3'
	Probe-Cy5[c]	5'-Cy5-AACCAACCCTTCGGGTAATCGGTG-BHQ2-3'

[a] 6-Carboxyfluorescein (FAM) fluoresces at 520 nm
[b] Texas Red fluoresces at 603 nm
[c] Cyanine5 fluoresces at 673 nm

centrifuged for 8 min at 2500 x g to pellet the blood cells, and the recovered serum or plasma was examined by darkfield microscopy.

***B. miyamotoi* positive blood samples.** Blood samples that contained *B. miyamotoi* were aliquoted in 200 µl amounts and stored as whole blood at -80°C or centrifuged at 2500 x g for 5 min to separate the plasma, and the plasma was also stored at -80°C. In addition, serum from additional samples in the Gundersen Health System Immunology Laboratory were retrieved and stored at -80˚C.

Concentration of spirochetes in blood samples. *B. miyamotoi* spirochetes in blood samples were concentrated as described previously (44). Briefly, the blood sample was centrifuged at 1,000 x g for 10 minutes. The plasma was then removed, transferred to a sterile tube, and re-centrifuged at 8,000 x g for 10 minutes. The supernatant was discarded, and the pellet was then used as inoculum.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Borrelia* spp. proteins were analyzed by SDS-PAGE using standard procedures. Briefly, 7.5 µg amounts of sonicated spirochetes were loaded into individual wells of a 10%-20% polyacrylamide gel (Criterion ™ TGX™ precast gels, Bio-Rad, Hercules, CA), and the proteins were separated by running in an electrophoresis chamber (Criterion ™ Cell, Bio-Rad) at 200V for 1 hour. The protein bands were then visualized by staining with Coomassie brilliant blue R-250. The sizes of the proteins were approximated by comparison with a high range rainbow molecular weight marker (GE Healthcare, Pittsburgh, PA).

Western blotting. Briefly, the spirochetal proteins were separated by SDS-PAGE as described, and then transferred to a polyvinylidene difluoride (PVDF)

membrane (PerkinElmer Life and Analytical Sciences, Boston, MA) by electrophoresing overnight at 10 V. The membrane was then cut into strips and placed in blocking buffer (phosphate-buffered saline, pH 7.2 containing 0.1% Tween 20 and 1% bovine serum albumin) for one hour at room temperature with gentle rocking. Following incubation, the strips were washed in PBS-T before adding human serum diluted 1:200 with blocking solution, and the strips were then re-incubated with gentle rocking for one hour at room temperature. The strips were then washed with PBS-T and horseradish peroxidase-labeled (HRP) goat anti-human IgG or IgM (Kirkegaard & Perry Lab Inc., Gaithersburg, MD) diluted 1:15,000 with PBS-T was added to the strips prior to incubating for an additional hour at room temperature. Strips were subsequently washed with PBS-T before developing by using the TMB membrane peroxidase substrate system (Kirkegaard & Perry Lab Inc.). A serum from a patient with no history of tick-borne illness was used as a negative control to determine when to stop developing the strips and a serum from an individual with Lyme disease was used as a positive control to confirm the ability to detect relevant band reactivities.

C6 peptide. The C6 peptide (49) was commercially synthesized (Genscript, Piscataway, NJ) by biotinylating the amine-terminal end by HBTU (2[1H-benzotriazole-1-y]-1,1,3,3-tetra-methyl-uronium hexafluorophosphate) activation and purifying the peptide by high-pressure liquid chromatography. The composition was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry.

C6 enzyme linked immunosorbent assay (ELISA). Antibodies specific for the C6 peptide were detected by ELISA using a modification of a previously-described method (50). Individual wells of microtiter plates (Immunolon 2 HB; Thermo

LabSystems, Franklin, MA) were coated with 100 µl (4.0 µg/ml) of streptavidin (Pierce, Rockland, IL) in 0.05 M carbonate coating buffer and incubated overnight at 4°C. Plates were washed with phosphate-buffered saline (pH 7.2) that contained 0.1% Tween 20 (PBS) to remove excess streptavidin and 200 µl of PBS that contained 1% BSA and 1.0 µg/ml of C6 peptide was added to each well prior to incubating the plate for one hour at 22°C. After incubation, 100 µl of immune serum diluted 1:200 with PBS were added to individual wells, and the plates were incubated at room temperature for one hr. After incubation, the plates were washed with PBS prior to adding 100 µl of horseradish peroxidase-labeled goat anti-human IgM diluted 1:20,000 or IgG diluted 1:30,000 (Kirkegaard & Perry, Gaithersburg, MD) in PBS, and the plates were re-incubated for one hour at 22°C. One hundred microliters of 0-phenylenediamine dihydrochloride substrate (Sigma-Aldrich) was then added to each well prior to an additional incubation for 30 min at 22°C. The reactions were stopped by adding 1N H_2SO_4, and the optical density (OD_{490}) was determined by spectrophotometry (VersaMax; Molecular Devices, Sunnyvale, CA). Samples that yielded OD_{490} values $\geq$ 0.200 above those obtained using a normal serum control were considered positive.

RESULTS

Ability of MKP-F to recover *B. miyamotoi* HT31. It was first necessary to confirm the ability of MKP-F medium to support the growth of *B. miyamotoi* HT31. As an initial evaluation, therefore, I inoculated separate 3 ml-volumes of culture medium with varying amounts (10^0, 10^1,10^2, 10^3, 10^4, or 10^5/ml) of the spirochetes, and then examined the cultures daily to determine the ability of the medium to recover the organisms from the various inoculums. In addition, I quantified the numbers of spirochetes in the cultures that yielded organisms to determine the incubation time necessary for the concentration of spirochetes to double (eg. generation time). Motile spirochetes were detected after incubation for 2 weeks when the number of organisms in the original inoculum was $\geq 10^3$ organisms per milliliter, but viable spirochetes were not recovered if the MKP-F was inoculated with fewer organisms (Table 2). In addition, the spirochetes replicated slowly, since at least 2 days of incubation was necessary for the concentration of organisms to double (Fig. 1). The findings therefore showed the standard MKP-F supported the growth of the relapsing fever spirochetes, but recovery was dependent on inoculating the medium with a relatively large number ($\geq$1000/ml) of organisms, and the replication rate appeared less than ideal. Therefore, an unimproved MKP-F medium could be considered less than ideal for successfully recovering *B. miyamotoi* from an infected patient.

Effects of major ingredients on the efficacy of MKP-F. To improve the efficacy, I initially evaluated whether removing or changing the concentration of several

TABLE 2 Detection (+/-) of motile spirochetes in MKP-F after inoculation with varying amounts of *B. miyamotoi* HT31. A positive (+) detection was made after microscopically examining two wet mount slides per concentration and seeing motile spirochetes.

		Number of spirochetes/ml inoculated					
		10^0	10^1	10^2	10^3	10^4	10^5
Incubation time (days)	6	-	-	-	-	-	+
	8	-	-	-	-	-	+
	10	-	-	-	-	+	+
	12	-	-	-	-	+	+
	14	-	-	-	+	+	+

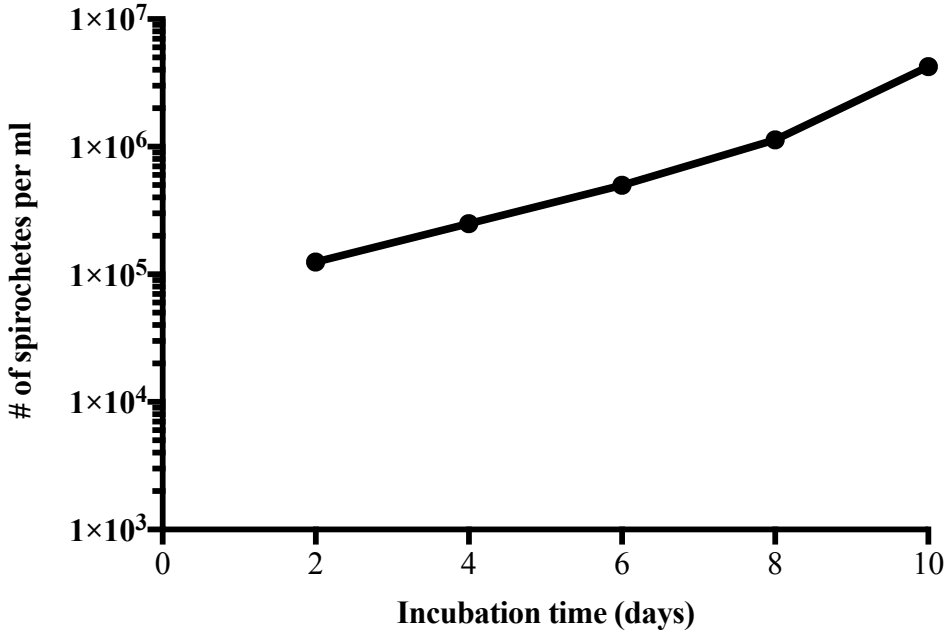

FIG 1 Concentrations of *B. miyamotoi* HT31 in MKP-F medium over time after inoculation with 10^5 spirochetes/ml. Counts were calculated using dark-filed microscopy and a Petroff Hausser counting chamber to find the number of spirochetes per ml.

major ingredients would affect the growth rate of the spirochetes. The effects of each modification were first evaluated by duplicate wet mounts and viewed at 400X with dark field microscopy. The number of spirochetes per 25 fields was then recorded to provide a preliminary evaluation of the effect. I first determined the impact of varying the manufacturer or fractionation procedure of the bovine serum albumin fraction V (BSA) on the replication and neither variable increased the growth rate compared to original MKP-F medium that was prepared with a Boval product. In fact, the incubation time necessary for the concentration of spirochetes to double in the preparations that contained BSA from Sigma was each significantly reduced, even if the BSA was recovered by using different manufacturing procedures (Fig. 2).

Next, the gelatin was removed from the culture medium to change the viscosity of the medium, and the growth rate obtained using the traditional MKP-F or the medium without gelatin was compared. The spirochetes in the MKP-F medium without gelatin replicated significantly faster (Fig. 3). In fact, the concentration of spirochetes in the MKP-F without gelatin was approximately doubled every 24-hours. Based on this finding, I then evaluated whether adding varying concentrations of human serum to the MKP-F medium would also result in an enhanced growth rate. The organisms again replicated rapidly in the MKP-F without gelatin, but the final concentration of viable spirochetes was increased even further if the culture medium also contained 10% human serum (Fig. 4). In contrast, however, the growth rate decreased significantly if the medium contained greater amounts of human serum. I therefore finally modified the MKP-F by eliminating the gelatin and adding a 10% volume of human serum, and the modified medium was designated as MKP-H.

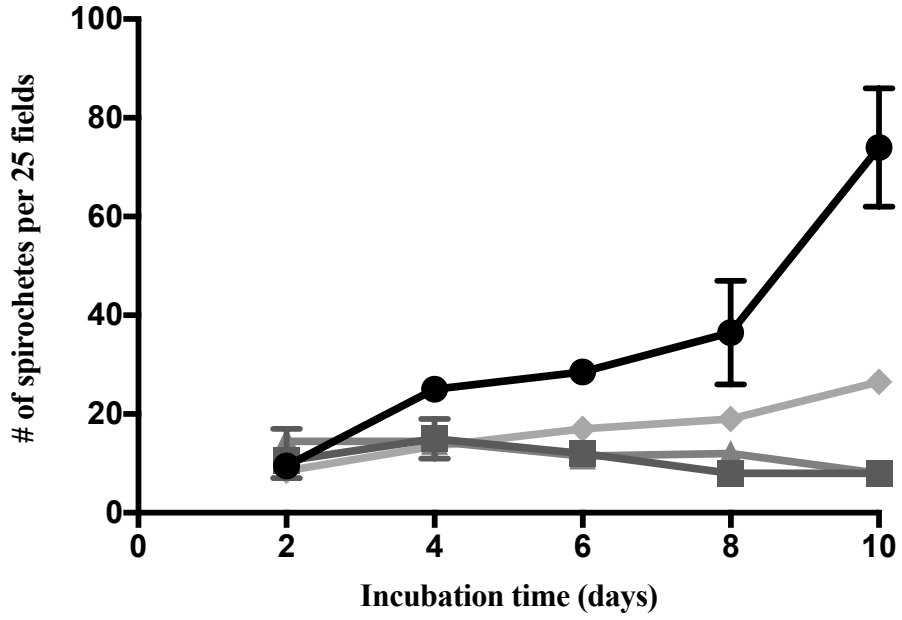

FIG 2 Growth rate of *B. miyamotoi* HT31 in MKP-F made with Boval fraction V BSA (●), Sigma lyophilized BSA (■), Sigma cold EtOH fractionated BSA (▲), or Sigma heatshock BSA (♦). Duplicate counts were made using dark-filed microscopy to count the number of motile spirochetes per 25 fields at 400X. Error bars indicate the range of duplicate counts.

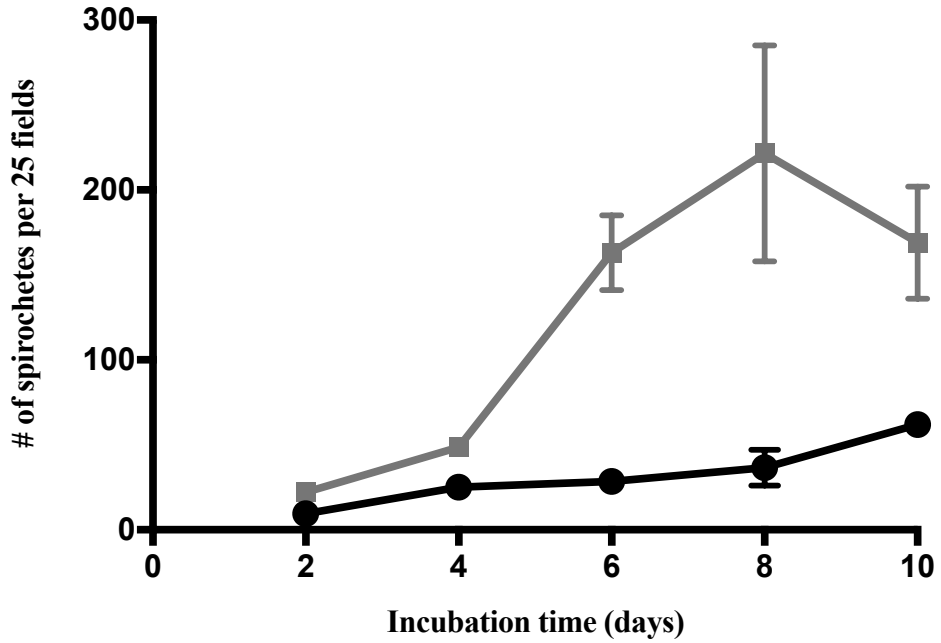

FIG 3 Growth rate of *B. miyamotoi* HT31 in MKP-F with (•) or without (■) gelatin. Duplicate counts were made using dark-filed microscopy to count the number of motile spirochetes per 25 fields at 400X. Error bars indicate the range of duplicate counts.

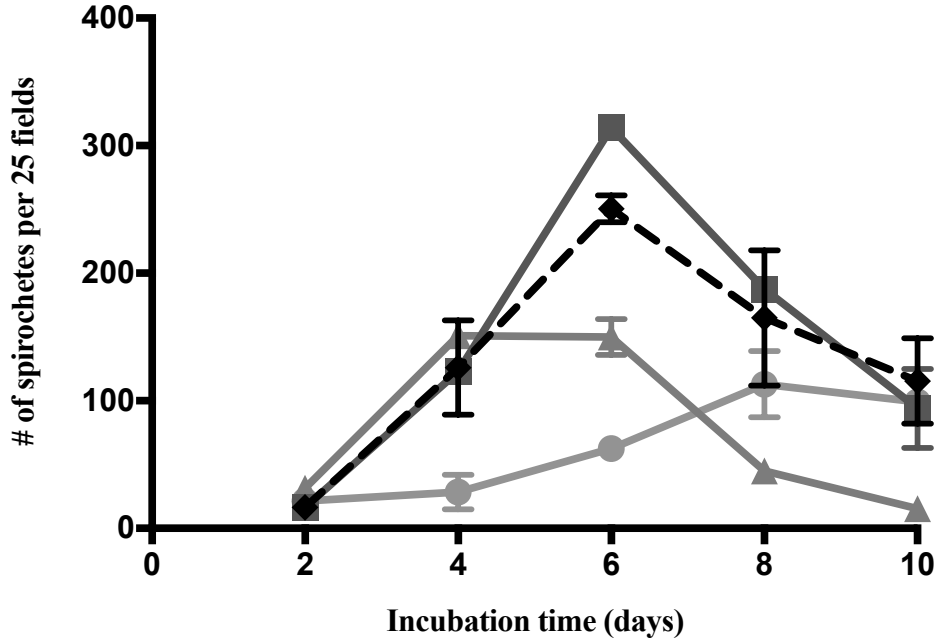

FIG 4 Growth rate of *B. miyamotoi* HT31 in MKP-F w/o gelatin (-♦-), MKP w/o gelatin 10% human serum (■), MKP w/o gelatin 30% human serum (▲), or MKP w/o gelatin 50% human serum (●). Duplicate counts were made using dark-filed microscopy to count the number of motile spirochetes per 25 fields at 400X. Error bars indicate the range of duplicate counts.

Effects of environmental factors on the efficacy of MKP-H. As a final evaluation, I examined the effects of pH and atmospheric CO_2 on the growth rate obtained using the MKP-H medium by evaluating growth in the formulation at the traditional pH of 7.4 and after adjusting the pH to 6.6 or 8.2. Increasing the pH to 8.2 had little effect on the growth rate and decreasing the pH to 6.6 was obviously deleterious (Fig. 5). In addition, there was no effect on growth, even when the CO_2 concentration was increased to 6% (Fig. 6). Therefore, the pH or level of atmospheric $CO2_2$ was not adjusted.

Increased efficacy of MKP-H. As final confirmation of the increased efficacy of the MKP-H, I finally examined the ability of the MKP-H medium to recover spirochetes from varying concentrations of *B. miyamotoi* HT31 and re-evaluated the growth rate, and the improved efficacy was striking. Viable spirochetes were now detected after inoculation with as few as one spirochete/ml (Table 3), and the concentration of organisms doubled after approximately 24 hours (Fig. 7).

Ability of MKP-H to recover spirochetes from human blood. The ability of the MKP-H medium to recover viable HT31 spirochetes from EDTA-containing blood was confirmed by spiking freshly obtained blood samples (8 ml) with either 10^0 or 10^3 HT31 spirochetes/ml, incubating the samples at room temperature, and removing 2 ml aliquots after 3, 6, or 12 hrs. The aliquots were then processed as described in the methods and materials, and the pelleted organisms were transferred to fresh MKP-H medium and examined weekly by darkfield microscopy for viable spirochetes. Recovery from an inoculum of approximately one organism/ml was inconsistent, but viable spirochetes were recovered consistently from the blood sample inoculated with at least 10^3

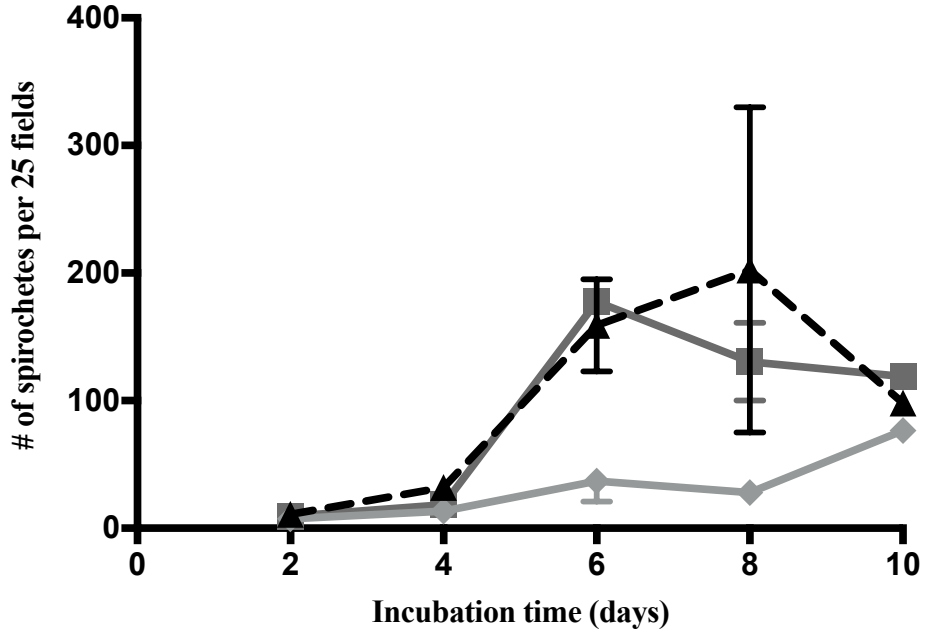

FIG 5 Growth rate of *B. miyamotoi* HT31 in traditional pH 7.4 MKP-H (-▲-) or after adjusting the pH to 6.6 (♦) or 8.2 (■). Duplicate counts were made using dark-filed microscopy to count the number of motile spirochetes per 25 fields at 400X. Error bars indicate the range of duplicate counts.

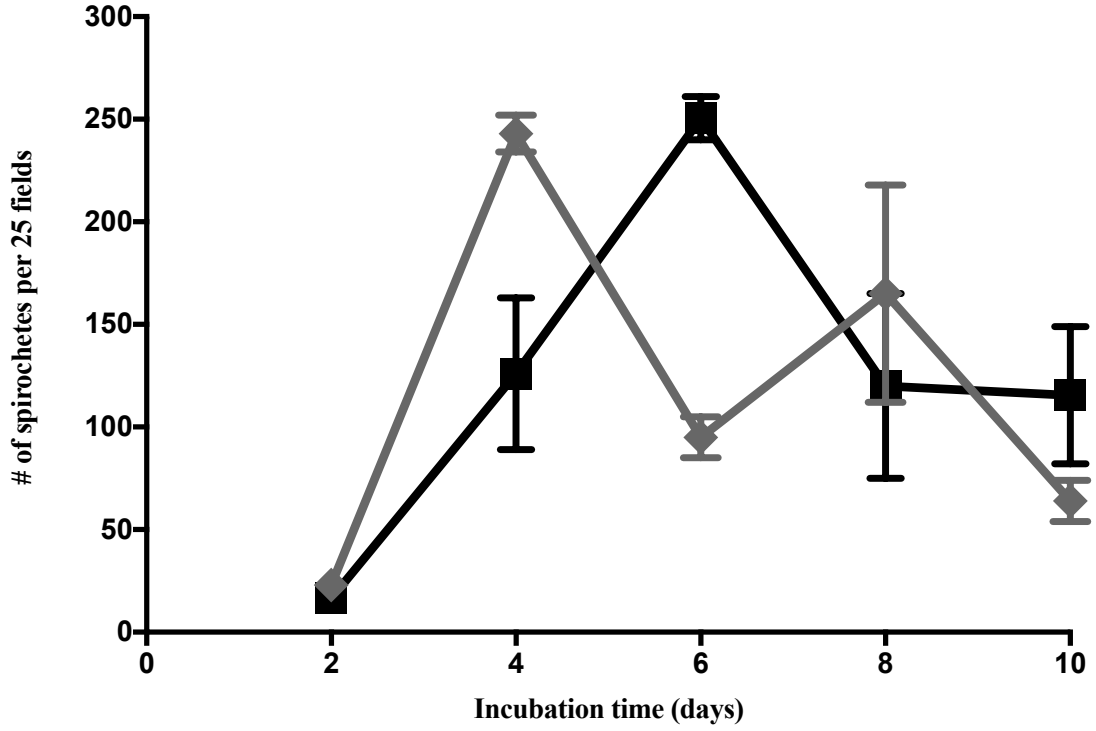

FIG 6 Growth rate of *B. miyamotoi* HT31 in MKP-H after incubation with (♦) or without 6% CO_2 (■). Duplicate counts were made using dark-filed microscopy to count the number of motile spirochetes per 25 fields at 400X. Error bars indicate the range of duplicate counts.

TABLE 3 Detection (+/-) of motile spirochetes in MKP-H after inoculation with varying amounts of *B. miyamotoi* HT31. A positive (+) detection was made after microscopically examining two wet mount slides per concentration and seeing motile spirochetes.

Incubation time (days)	Number of spirochetes/ml inoculated			
	10^0	10^1	10^2	10^3
6	-	-	-	-
8	-	-	-	-
10	-	-	-	+
12	-	-	+	+
14	-	+	+	+
16	+	+	+	+

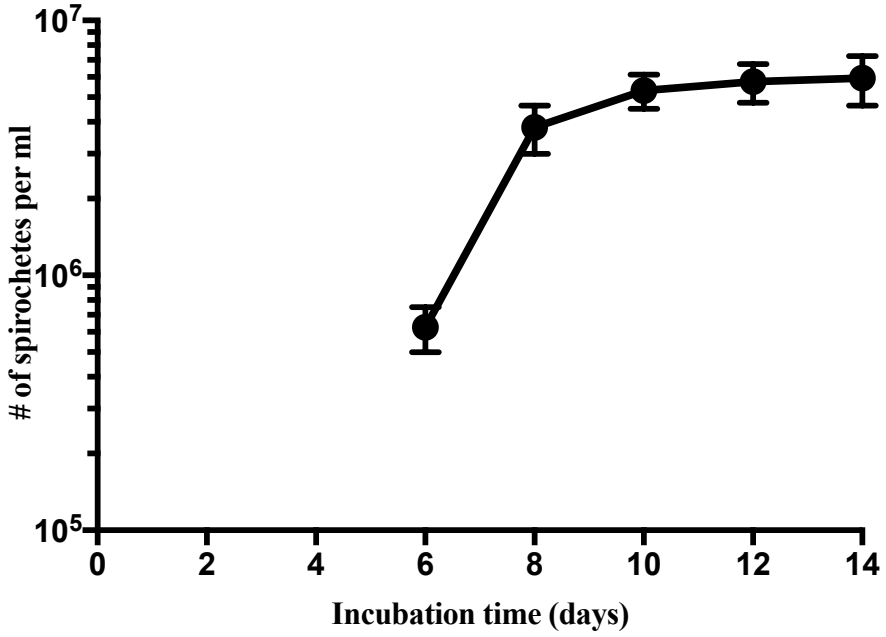

FIG 7 Growth rate of *B. miyamotoi* HT31 in MKP-H after inoculation with 10^4 spirochetes/ml. Duplicate counts were made using dark-filed microscopy and a Petroff Hausser counting chamber to find the number of spirochetes per ml. Error bars indicate the range of duplicate counts.

organisms/ml, even after the spirochetes had been exposed to the EDTA contained within the vacutainer tube for up to 12 hours (Table 4).

Recovery of relapsing fever spirochetes from infected human blood samples. The collective findings provided impetus to attempt to use the MKP-H medium to recover *B. miyamotoi* from blood samples from Gundersen Health System patients, especially since the spirochetes were detected by darkfield microscopy in several PCR-positive blood samples. Therefore, the infected blood in those samples was likely to contain at least 10^4 organisms/ml, since it is generally-accepted that the concentration of microorganisms in a blood sample had to be at least 10^5 organisms/ml for reliable detection by microscopy. In fact, during the course of the studies to optimize the culture medium, blood samples from three patients with relapsing fever from *B. miyamotoi* confirmed by PCR (two were also confirmed by visual detection of motile spirochetes) had been stored in 200 µl-amounts at -20°C. The MKP-H medium was therefore inoculated with an aliquot of each archived blood sample. However, despite incubating the samples for 6 weeks, viable spirochetes were never detected. In addition, the MKP-H medium was inoculated with fresh blood samples from two additional patients that were positive for *B. miyamotoi* by PCR, and even these fresh blood samples failed to yield viable spirochetes. The failure to recover the spirochetes, even from the blood with higher concentration of viable spirochetes that could be detected by microscopic examination, provided strong evidence that, despite the improved efficacy for cultivating HT31 spirochetes, the MKP-H medium was still lacking an ingredient that was critical for recovering *B. miyamotoi* sl.

TABLE 4 Recovery (+/-) of viable spirochetes from blood samples spiked with *B. miyamotoi* HT31 by using culture medium MKP-H. A positive (+) detection was made after microscopically examining two wet mount slides per concentration and seeing motile spirochetes.

		Culture incubation time (wks)			
Inoculation (organisms/ml)	Time (hrs) in blood	1	2	3	4
10^0	3	-	-	-	-
	6	-	+	+	+
	12	-	-	-	-
10^3	3	+	+	+	+
	6	-	+	+	+
	12	+	+	+	+

Detection of anti-*B. miyamotoi* antibodies in immune sera and cross-reactivity with *B. burgdorferi*. Despite the failure to recover *B. miyamotoi* from the infected human blood, a preliminary evaluation of the antibody responses in 16 archived immune sera from patients with *B. miyamotoi* PCR-positive blood by Western blotting using the HT31 isolate was performed. Interestingly, each immune serum contained IgM antibodies that bound an approximately 75 kDa protein and IgG antibodies that bound an approximately 52 kDa protein. However, the reactive bands were also detected when normal human serum was probed, so the most likely explanation for these antibody responses was an error in methodology. Despite this probability, however, the antibody responses were still detected, even after several repeated efforts. Therefore, reactivities against these proteins were not considered when the reactivities detected in the immune sera from patients with *B. miyamotoi* infections were evaluated.

There were, however, additional antibody reactivities that appeared likely due to infection with *B. miyamotoi*. Most notably, 5 (36%) sera contained IgM (Fig. 8, panel A) or IgG antibodies (Fig. 8, panel B) that bound multiple proteins, but there was no consistent banding pattern apparent. Therefore, additional studies to discriminate specific antibody response associated exclusively with infection with *B. miyamotoi* remain necessary. In addition, when the reactive sera were also tested for antibodies that bound *B. burgdorferi* proteins, IgM and IgG antibodies that bound multiple proteins were also detected (Fig. 9). Additionally, when the reactive sera were tested for antibodies that bound the C6 protein, which is regarded as a highly specific (> 97%) marker of exposure to *B. burgdorferi*, 3 (60%) of 5 reactive immune sera also contained significant levels

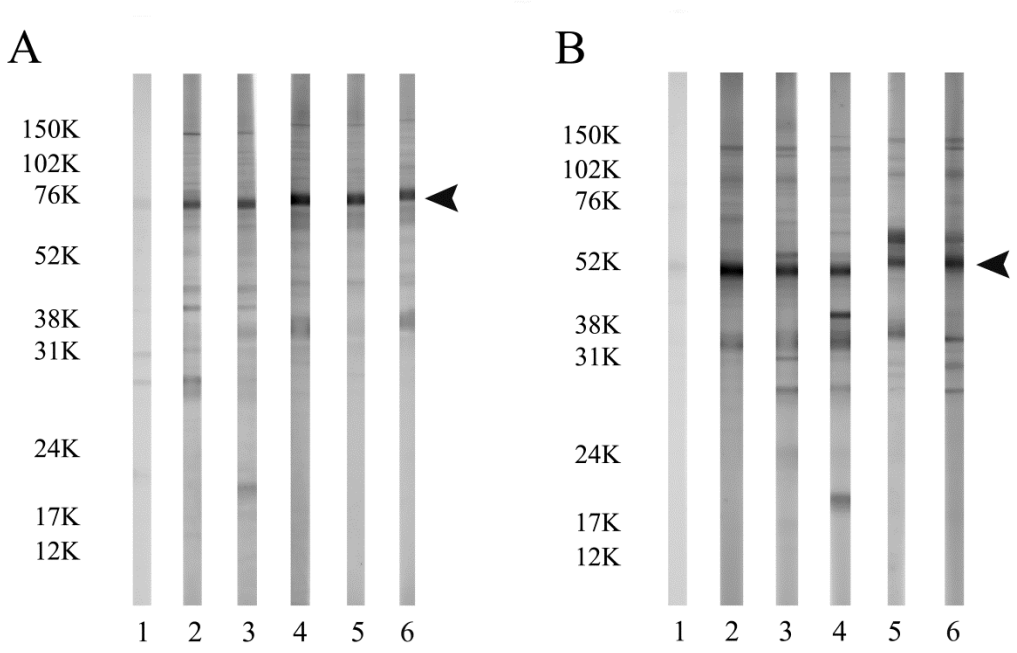

FIG 8 IgM (panel A) or IgG (panel B) antibodies detected in immune sera from patients with relapsing fever by using Western blotting with *B. miyamotoi* HT31. Lane 1 depicts the normal serum control. Arrows indicate bands that were not considered due to methodology errors.

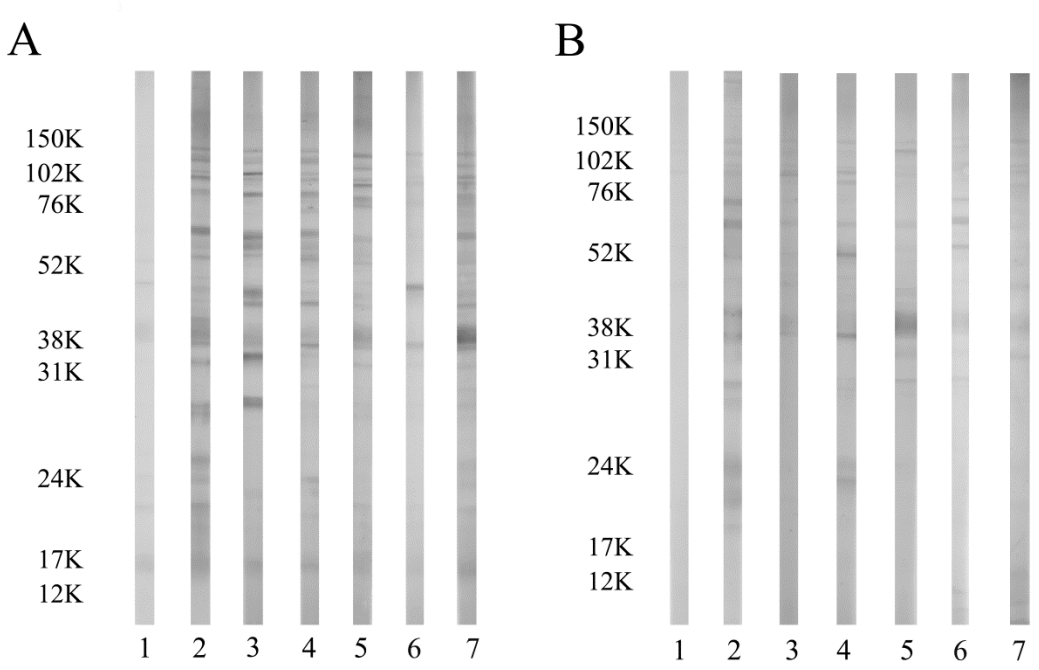

FIG 9 IgM (panel A) or IgG (panel B) antibodies detected in immune sera by using Western blotting with *B. burgdorferi* 297. Lanes 1a and 1b are the normal serum controls. Lanes 2a and 2b are positive controls using serum from a patient with a *B. burgdorferi* infection. Lanes 3-7 are the antibodies detected in immune sera from patients infected with relapsing fever.

of antibodies that bound C6 (OD_{490} values $\geq$ 0.200 above normal serum). The collective results, therefore, confirmed that discriminating infection with either relapsing fever or Lyme disease spirochetes will be confounded significantly by cross-reactive antibody responses and therefore additional studies to characterize antibody responses specific for each illness remain necessary.

DISCUSSION

The recent discovery that *I. scapularis* ticks, thought to be associated exclusively with transmission of Lyme disease-causing spirochetes, also transmit the relapsing fever spirochete *B. miyamotoi* is significant. In addition, researchers (22) confirmed that *I. scapularis* ticks captured near La Crosse, Wisconsin harbor the relapsing fever spirochetes and documented several human cases of relapsing fever in residents from this geographic region. This highlighted the necessity of research efforts to gain better understanding of this emerging pathogen. For example, one significant issue of concern is that, because relapsing fever and Lyme disease spirochetes likely share many proteins, the antibody responses induced during infection with either organism could be expected to cross-react and thus negatively impact the ability to accurately differentiate the infections. Therefore, studies to discriminate antibody responses specific to infection with *B. miyamotoi* are needed.

Studies with this as a goal, however, have not been performed to date because, while the spirochetes have been successfully recovered from infected ticks (17, 41) and human patients from Russia (44), the organisms have not been recovered from a human patient in North America. This is especially significant because the expression of proteins by Lyme disease spirochetes that colonize ticks or mammalian hosts (39, 40, 51) have typically varied greatly and *B. miyamotoi* is likely to act similarly. In fact, the inability to recover *B. miyamotoi* from a US patient by using the same laboratory culture medium used successfully to recover the organisms from ticks and Russian patients

provides strong support for this possibility. Therefore, the accuracy of studies designed to characterize antibody responses that occur during human infection may be dependent on the antigenicity of the spirochete, and the source of the organism may have a great impact on antigenicity.

In this study, my ultimate goal was to expedite future studies by successfully recovering a *B. miyamotoi* spirochete from an infected human patient. As a starting point, I evaluated the ability of the traditional MKP-F culture medium to support the growth of the tick isolate HT31, since the medium could be used to successfully recover *B. miyamotoi* isolates from infected ticks (41), and then determine whether changing or manipulating the concentrations of several essential ingredients would increase the efficacy of the medium. In my initial experiments, I confirmed that HT31 spirochetes survived and replicated in MKP-F, but successful recovery depended on an inoculum of $\geq 10^3$ organisms/ml, and the replication rate of the cultivated spirochetes was slow.

Therefore, it was necessary to improve the efficacy, so I initially evaluated whether using different manufacturing processes or commercial suppliers of albumin, which is a major ingredient in MKP-F, would have an effect and showed that the source and manufacturing process profoundly affected efficacy. In fact, the albumin that was initially used provided the best results. This finding corroborated previous work (47) that showed the quality of the albumin was a critical component in the culture medium used to propagate Lyme disease spirochetes.

I then showed that eliminating gelatin and adding a 10% volume of human serum significantly decreased the inoculum size necessary to recover the spirochetes and also increased the replication rate of the organisms. Likely explanations for the increased

efficacy were that culture medium without gelatin provided an environment that more closely matched the bloodstream and human serum provided an essential growth nutrient. However, neither manipulating the pH or changing the atmospheric CO_2 levels affected the growth rate, which was unexpected since *B. miyamotoi* are microaerophilic and other researchers (42, 48, 52) reported that manipulating these variables, especially CO_2 levels, significantly increased the growth rate of other *Borrelia* spp.

However, eliminating the gelatin and adding human serum had apparently increased the efficacy sufficient to attempt isolating *B. miyamotoi* from infected human blood. Therefore, as a final evaluation I determined whether the improved MKP-H medium reliably recovered HT31 spirochetes from spiked fresh blood samples that contained EDTA. This was important step because blood samples obtained from Gundersen Health System patients are collected in vacutainer tubes that contain EDTA to prevent the blood from clotting by supplying chelating factors (eg. calcium) that are coincidentally toxic to microorganisms. Thus, it was not surprising that small numbers of organisms were killed by the EDTA, as an inoculum concentration of at least 10^3 spirochetes per ml was necessary to reliably recover viable organisms.

However, despite the decrease in efficacy, the finding was encouraging because, while I focused my efforts on improving the culture medium, I had identified three blood samples from patients with *B. miyamotoi* infection and two samples contained at least one viable spirochete per darkfield microscopic field. Therefore, the concentration of spirochetes in these samples was likely at least 10^4 organisms/ml since it is generally believed that concentrations of microorganisms < 10^5/ml are not reliably detected by microscopy. One additional major drawback, however, was that the blood samples were

also stored at -20ºC while I was performing other experiments, which could also be lethal. Despite this, I thawed the blood and inoculated the MKP-H medium, but the medium failed to recover viable spirochetes and I ultimately confirmed the spirochetes were indeed dead by inoculating SCID mice with the infected blood and showing that spirochetes were not present in the blood by both microscopic examination or PCR (data not shown). Therefore, successful recovery was likely dependent on using fresh blood samples.

I addressed this concern by attempting to recover *B. miyamotoi* spirochetes from fresh blood samples collected from two additional patients identified after I had fresh MKP-H medium available and each failed to yield viable spirochetes. This was especially disappointing because viable spirochetes could easily be detected in one of the samples by microscopy and another research group (44) concurrently reported successfully recovering a *B. miyamotoi* isolate from human patients residing in Russia. Moreover, the researchers recovered the spirochetes using traditionally-prepared (41, 44) MKP-F culture medium. These findings, however, provided strong evidence that the MKP-H medium still lacked a critical ingredient necessary for cultivating *B. miyamotoi* spirochetes from an infected US patient. As further support, researchers at the Centers for Disease Control and Prevention have also to date failed to recover the spirochetes from a US patient, despite an ongoing intensive effort to also develop an appropriate laboratory culture medium (personal communication).

The failure to recover *B. miyamotoi* from an infected human ultimately made my final goal of accurately characterizing the antibody responses by using a human isolate impossible. As an alternative, therefore, I analyzed the antibodies in serum samples from

16 patients infected with the relapsing fever spirochete and showed that 5 (31%) contained IgM and IgG antibodies that bound multiple proteins. However, the antibody response was without an apparent pattern of reactivity, and each reactive serum contained both IgM and IgG antibodies that also bound multiple *B. burgdorferi* proteins. In fact, three (60%) of the 5 most reactive sera also contained anti-C6 antibodies, which are widely recognized as a highly specific marker of exposure to *B. burgdorferi* (49, 50). Therefore, the patients may have been concurrently infected with Lyme disease spirochetes, or previously exposed to the organisms, or there was considerable cross-reactivity that could be expected to significantly hinder efforts to discriminate the illnesses. In support for the latter, a recent study (53) also showed anti-C6 antibodies in immune sera from most patients with *B. miyamotoi* infection and no previous diagnosis of Lyme disease. Therefore, the collective findings highlighted the high likelihood that laboratorians will have difficulties providing serologic findings sufficient to provide accurate discrimination of infection with either Lyme disease or relapsing fever spirochetes. Moreover, this will be especially problematic in regions like La Crosse, Wisconsin that are well-recognized highly endemic foci for Lyme disease.

In summary, the efficacy of the traditional MKP-F medium used to cultivate *B. miyamotoi* can be improved significantly by eliminating gelatin and adding human serum. However, the modified MKP-H medium will likely still fail to support the growth of *B. miyamotoi* from local infected patients because the medium still lacks an unknown critical growth requirement. In addition, the findings confirmed that patients infected with *B. miyamotoi* produce antibodies that also bind multiple *B. burgdorferi* proteins, which highlights the necessity of obtaining a human *B. miyamotoi* isolate so that the

antibody responses specific to the relapsing fever spirochetes can be more accurately characterized.

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