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Experimental infection of gnotobiotic calves with *Borrelia burgdorferi* and evaluation of antibody detection techniques

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by

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A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Microbiology, Immunology and Preventive Medicine
Major: Veterinary Microbiology (Preventive Medicine)

Approved:

Signatures have been redacted for privacy

Iowa State University
Ames, Iowa

1992

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GENERAL INTRODUCTION

Lyme borreliosis (Lyme disease) is a progressive, chronic, infectious disease that involves multiple organ systems and occurs in people and animals. The causative agent is a recently discovered spirochete, *Borrelia burgdorferi* that is transmitted through the bite of a tick.[28,31,32] The disease has been reported in at least 46 states, and is now the most common tick-vectored disease in the United States (U.S.).[46,98]

The significance of Lyme borreliosis has been realized on a national scale due to its rapid emergence and spread, as well as the clinical syndromes produced. The disease often goes undetected or misdiagnosed. The fact that many patients have experienced years of discomfort from the infection, and that treatment may not be curative, has led to intense research and education regarding various aspects of the disease.

Clinical manifestations in humans are varied but may follow a progression of events to include: a) Early local infection with erythema migrans (EM) and/or a flu-like illness, b) Acute disseminated infection involving cardiac abnormalities, neurological abnormalities, hepatitis and arthritis, and c) Chronic disseminated infection with neurological abnormalities, arthritis and acrodermatitis chronica atrophicans.[8,60,140,142] Lyme borreliosis in animals has been reported in dogs, cats, horses, and cattle and is suspected in sheep.[6,24,35,36,38,48,49,67,68,73,91,103,106,114,122,123,127] The clinical presentation in animals is not as well defined as in humans, but in general includes: fever, lethargy, anorexia, joint pain, lameness, joint swelling, behavioral changes, seizures, uveitis, arthritis, abortion and encephalitis.[38,39,40,73,101,102,148] These clinical aspects do not represent the disease of any single animal species. The importance and occurrence of borreliosis in animals is increasing. Owners of pets in areas endemic for the infection may be at increased risk of exposure to disease carrying ticks, and disease in animals of economic importance may affect their value.

There are many problems associated with Lyme borreliosis. The first and foremost is correct diagnosis. Lyme borreliosis has been referred to as the "Great Imitator," in that it often resembles other diseases.[118] The clinical picture can be varied in both humans and animals. One person may present with classical defined symptoms such as EM and another with flu-like illness and no history of a rash. Early diagnosis is a key for rapid treatment with antibiotics. Prolonged infection can produce a debilitating syndrome in some patients, either human or animal and make treatment difficult. Ideally, diagnosis would include demonstration of the spirochete in body tissues or fluids, but isolation of the organism from an infected host is extremely difficult and negative findings are unreliable. The need for specialized culture media, the low number of circulating spirochetes, and the time requirements for growth make bacteriologic culture impractical for clinical diagnosis. As a result, serologic tests are utilized as a diagnostic tool in determining Lyme borreliosis.

There have been several protocols developed for detecting antibodies against *B. burgdorferi*. However, evaluation of the results is difficult due to the lack of standardization and the use of a variety of antigens. Commonly used methods are indirect immunofluorescent antibody (IFA) tests,[109,129] enzyme immunoassays (EIA) and immunoblot techniques.[22,75,76,87,89]

This thesis is composed of two sections. Part I is limited to experimental infection of germ free calves with *B. burgdorferi*. The study was designed to assess the IgG class immune response and potential clinical manifestations associated with Lyme borreliosis without the presence of additional microbiological or environmental factors. An Immunofluorescent Assay (IFA) was used exclusively throughout Part I for detection of IgG class antibody response against *B. burgdorferi*. IgM detection was not part of this study.

Information and materials gained in Part I were used for control and comparison purposes in the second section of this thesis. Part II was designed to evaluate immunodetection techniques and determine their suitability for use in performing serological surveys of bovine populations

for prevalence of exposure to *B. burgdorferi*. Enzyme Linked Immuno Assay (ELISA), Western Blot, Dot Blot, and IFA immunodetection techniques using enriched or purified extracted flagellar proteins, whole cell preparations, synthetic amino acid sequences from the flagellar gene were performed.

LITERATURE REVIEW

Background Review

The Lyme disease syndrome was first characterized in the U. S. by Dr. Allen Steere at Yale University in 1975.[140] The discovery was the result of a report, and subsequent investigation of an unusually high incidence (100 times the normal rate), of juvenile rheumatoid arthritis in the area of Lyme, Connecticut. The clinical aspects of the outbreak were not totally consistent with all features of juvenile rheumatoid arthritis, and interviews with the victims revealed that a rash, thought to be associated with an insect bite, had developed early in some cases. The rash or lesion appeared to be consistent with that of erythema migrans (EM) which was known in Europe. The condition, described by Afzelius in 1909, was thought to be a result of a tick bite.[1] EM was described in Wisconsin by Rudolph J. Scrimenti in 1970 but was not associated with arthritis.[134]

In 1982, the etiologic agent for Lyme disease, *B. burgdorferi*, was identified by Dr. Willy Burgdorfer at the Rocky Mountain Laboratory of the National Institutes of Health.[28] The agent was isolated from the blood, cerebro-spinal fluid and the skin of patients with the disease.[17,139] Since the discovery of Lyme disease and the causative agent, a great deal of information has been learned about its epidemiology, treatment, detection, specific disease symptoms, prevention, and diagnosis. It is known that there are at least two tick vectors in the U. S., *Ixodes dammini,* and *I. pacificus*.[2,30,32,125] Isolations have been made from other arthropods, but their ability to efficiently transmit the disease remains unclear.[107] Animals serve as reservoirs, with the white-footed mouse, *Peromyscus leucopus*, identified as the principal host reservoir. The life cycle of the *Ixodes* tick has been outlined in detail, and studies on the transmission mechanisms of the tick have been completed. Migratory birds have been implicated as being vehicles for dispersing the infected ticks, helping to explain the expanding geographic spectrum

of Lyme disease.[5,149] In addition to disease in human patients, clinical cases have been reported in dogs, cats, horses, cows and goats. Wildlife, such as deer, raccoons and various rodents do not seem to be clinically affected, but do develop immune responses.

Causative Agent

The order Spirochaetales is composed of two families: Spirochaetaceae and Leptospiraceae. *B. burgdorferi* belongs in the family Spirochaetaceae along with the genus *Treponema* which also includes species which are pathogenic for animals and human beings. All species within the *Borrelia* genus are transmitted to vertebrates by hematophagous arthropods.[9]

Common morphological features shared by *Borrelia* and other spirochetes are an outer envelope, a protoplasmic cylinder with a peptidoglycan layer, cytoplasmic membrane enclosing the cytoplasmic contents, and periplasmic flagellae positioned between the outer envelope and the protoplasmic cylinder. The periplasmic flagellae are attached at each end of the cylinder and extend toward opposite ends and with exception to *Leptospira*, overlap each other.[80,86] *Borrelia burgdorferi* has 7-11 PF per termini. The flagellae are responsible for the characteristic motility of spirochetes in viscous environments.

The outer envelope is loosely attached and easily disrupted.[9] The chemical composition of the outer envelope is 42% protein, 50% lipid, and 4% carbohydrate.[53] The cell length ranges from 4-39 μm and a diameter of 0.18-0.25 μm. Length of the organism is an unreliable variable, as age of culture and nutritional composition of the medium are identified as factors which influence length.[9] Studies for the presence of lipopolysaccharides (LPS) reported conflicting results in that one study found endotoxin similar to the gram-negative rough form and the other found that the organism lacked polysaccharide. The use of two distinct procedures may explain the differences in results of these studies.[16,77,143]

Bacteriologic culture of *B. burgdorferi* can be accomplished in Barbour-Stoenner-Kelly (BSK) medium.[7,43,92,141] Growth requirements are specific and preparation of the medium is detailed. The organism is microaerophilic and in BSK medium divides by binary fission approximately every 12 hours. Isolation and subsequent culture from patients is difficult. Recovery from infected ticks is more readily achieved. Low numbers of organisms present in the patient vs. a more concentrated population in the tick are probable factors. The organism has been shown to lose pathogenicity in culture which may be due to the loss of certain plasmids.[133] Antigenic variation occurs more often with repeated *in vitro* passage and probably involves several factors such as environmental conditions and nutrient composition of the medium.[116,133,150]

Vectors of Lyme Borreliosis

In the eastern and midwestern United States, the deer tick, *I. dammini*, is considered to be the vector of *B. burgdorferi*. The identified vector for the western part of the Nation is *I. pacificus*. In Europe, the principal vector is known to be *I. ricinus*. Areas endemic for Lyme disease can yield ticks with high infection rates.[2,30,32] The organism has also been found associated with biting flies and mosquitoes, as well as other ticks such as *Dermacentor variabilis*, and *Amblyoma americanum*; however, their ability to maintain the organism or to transmit the disease is uncertain.[107]

The life cycle of ixodid ticks involves three stages. The eggs are deposited in the soil and hatch in early spring. The larval ticks emerge and feed in the late summer, then enter a molting period. The nymphs emerge the following spring and seek their second blood meal. Upon repletion, the nymphs molt to the adult stage. The adults find suitable hosts for their final blood meal before depositing eggs and dying, thus completing the two-year life cycle. The larval and nymphal stages of *I. dammini* are known to parasitize at least 29 species of mammals and 49 species of birds. The adult ticks have been recovered from 13 species of

mammals, including large domestic animals. The ticks in all stages are known to parasitize human beings.[3]

During ingestion of a blood meal containing spirochetes, some of the organisms migrate from the gut through intercellular spaces into the hemocoel of the tick as demonstrated by transmission electron microscopy.[18,126] Transmission by the ticks to hosts occurs during the salivation stage of the feeding period.[29,32,33,126] The duration of tick attachment and transmission efficiency are strongly correlated.[121] The longer a tick is attached and feeding, the greater the ability for transmission to occur. A public health consideration for prompt removal of ticks is indicated.

Immunology

The known immune response to *B. burgdorferi* in human beings is generally accepted to include humoral,[56,57,58] cellular,[60,114,142] and phagocytic components.[20,145] Antibody responses to the pathogen are similar to those of other bacterial infections. IgM class antibodies appear in the early stages of the disease with the peak occurring in the first three to six weeks.[58] The response is typically specific for the 41 kilodalton (kDa) molecular weight (mw) endoflagellar antigen. The IgM class antibody can be detected for months to years.[56,57,58] Antibiotic treatment may alter the early specific IgM response.[58]

Following the IgM response, a specific IgG response occurs to the same 41 kDa protein. This response can first be detected four to six weeks after onset of disease and may persist for months to years.[58,60] Following the initial response to the 41 kDa antigen, antibodies develop to multiple protein components of the organism.[56,58] Some of these components include 83-, 66-, 27-, and 15 kDa proteins. Even later, antibodies specific for the 75-,60-, 34-, 31-, 29-, and 17 kDa proteins develop.

This gradual evolution of antibody development to different proteins has not been proven to correlate with appearance of specific clinical manifestations of Lyme disease. Instead, the general consensus is that it seems to simply reflect the maturation of the humoral response over time. Delayed development of the response to specific protein components may be related to gradual presentation during the natural decomposition of the *Borrelia* cells.[114]

A cellular immune response occurs early in the disease and prior to a measurable IgM humoral response to include a period of increased suppressor activity, but was not evident in the late stages of active disease.[114,136] Natural killer cells are also increased in early infection but their function may be inhibited by the presence of spirochetes.[145]

During the course of the disease, phagocytes and *B. burgdorferi* can be found in proximity to each other. Macrophages are present in the EM lesion and polymorphonuclear leukocytes (PMN) are abundant in diseased joints. Phagocytosis of *Borrelia* cells has been demonstrated experimentally.[20] Purified outer surface protein A (OspA) and the flagellar protein have been shown to be chemotactic for human PMN. The observations were made with both PMN and monocytes with or without presence of opsonizing antibodies.[19]

Even with a full compliment of immune response, active infection may persist, indicating that antibodies are not able to eliminate the organism. There are also individuals who are asymptomatic but seropositive possibly representing successful suppression of the disease process.

Borrelia burdorferi has been shown to exhibit an efficient ability for adherence to and penetration into cultured endothelial cells.[55] *Borrelia* did not remain intracellular in the study and either emerged from or were degraded within the cell. Attachment to cells is thought to be dependent on bacterial protein(s)/cell membrane interaction. Invasion of the cells was shown to be dependent upon spirochete motility. The ability to invade cells may play an important role in understanding asymptomatic periods, persistent infection, and treatment.

Animal Studies

There have been several attempts to develop an animal model for the study of induced Lyme borreliosis. Experimental animal studies have included rabbits, hamsters, rats, dogs, and mice.[12,13,29,34,37,86,90,116,148] To date, no single animal model has been established that enables researchers to fully study the disease progression as it relates to humans. The most successful animal models have been studies with dogs and vaccine development.[46,148] Valuable information, however has been gained on various aspects of immunology, histopathological changes, infectivity, and transmission.

The first animals to be used for laboratory studies were rabbits.[18,29,90] Following inoculation of the organism, skin lesions developed. The lesions were not like the lesions observed in human beings, developing more as dermal inflammatory responses. Rabbits also did not develop any detectable disseminated disease and therefore were not considered adequate as an animal model. Ticks became infected after feeding on infected rabbits, indicating bacteremia occurred.

Syrian hamsters were the first animals used in experimental infection with recovery of the organism from the blood and other organs.[86] Hamsters inoculated intraperitoneally were spirochetemic within 24 hours. Spirochetes were recovered from the spleens, kidneys, testicles, livers, brains, and eyes. Isolations from the blood were intermittent, indicating a blood-borne phase can occur repeatedly or that numbers of circulating spirochetes were low. The first findings of diminished virulence of *B. burgdorferi* as a result of serial *in vitro* subculture was also indicated in this study.

In another study, normal Syrian hamsters were passively immunized with anti-*B. burgdorferi* antiserum produced in rabbits or hamsters.[139] The hamsters failed to become infected, ie, no spirochetes could be recovered. The same was not true when antiserum was given after challenge with the organism. Passive immunization was shown to be strain specific in that

antiserum produced from one strain did not protect against challenge with a strain from a different geographical location. Although the hamster has proven to be a valuable model for immunization and infection studies, clinical signs are lacking.

Neonatal (three-day-old) Lew\N rats developed arthritis 14 days after intraperitoneal challenge with *B. burgdorferi*.[13,115,116] Organisms were recovered from multiple organ systems. Challenge with killed spirochetes failed to produce any clinical disease. Other studies using varying aged rats determined that younger animals exhibited more severe manifestations.[13]

Mice have also been used in the search for an animal model.[12,130] Inoculation with a high culture passage isolate resulted in mild histopathological changes in various organs of several inbred mouse strains. Low *in vitro* passage strains of *B. burgdorferi* inoculated into severe combined immunodeficient (SCID) mice produced severe arthritis and in some, evidence of endocarditis.[130] Signs of disease were not noted with use of high-passage inocula of the same strain of organism. The immunological response to *B. burgdorferi* in laboratory rodents differs from that of humans, however there is a natural tendency for the spirochete to cause joint disorders in both. Mice respond early to OspA and OspB which is not the case in a human infection.[64,65] The need to utilize an immunocompromized animal host in order to observe disease is contrary to observations in humans.

PART I: EXPERIMENTAL INFECTION OF GNOTOBIOTIC CALVES WITH THE AGENT RESPONSIBLE FOR LYME BORRELIOSIS, *Borrelia burgdorferi*

LITERATURE REVIEW

Lyme Borreliosis in Cattle

Lyme borreliosis is recognized in cows.[6,36,38,,122,123,127] Cases of the disease are on the increase; however accurate documentation is not available since it is not a reportable disease. The area of highest incidence appears to be in the state of Wisconsin where its importance as a debilitating condition is being realized and where most research on bovine animals is being conducted. The primary tick vector, *I. damnini* is common in Wisconsin.

Experimental oral infection of mice with *B. burgdorferi* has been established [41] and *B. burdorferi* has been isolated from urine.[26,36,72,81] This suggests that Lyme borreliosis, like leptospirosis, may be spread through contaminated urine contact with mucous membranes. In addition, spirochetes may be present in unpasteurized milk and may serve as a vehicle for oral infection.[123] Transplacental transmission is suspected in animals as it is in humans.[94,131]

Diagnosis of Lyme borreliosis in cattle, as in humans and other animals, is difficult and may be considered controversial. Many cows have serum antibodies against *Borrelia* when tested using current testing techniques, even though the cattle are from areas known to be free of known tick vectors, suggesting poor specificity of tests, cross-reacting antibodies, or possible alternate transmission routes or vectors. Animals often have elevated titers to *Borrelia* with no evidence of clinical signs. Seropositive herds sometimes have an array of clinical signs and other positive herds do not have any clinically affected animals.[36,122]

Clinical observations in cattle include fever, lameness, single or multiple swollen joints, arthritis, laminitis, weight loss and decrease in milk production. Abortions and stillbirths, although not proven, are suspected as being caused by *B. burgdorferi*.[36,40,94,123]

In a Wisconsin study,[36] 282 of 430 cows with signs thought to be caused by *B. burgdorferi* infection had serum antibodies against the spirochete as determined by indirect

immunofluorescent (IFA) tests. In addition, antibodies were detected by IFA tests in five of ten synovial fluids, two of three samples colostrum samples, zero of 44 milk samples, and one serum sample from an aborted fetus. October and May were two peak months for positive samples. Not all of these animals presented with clinical signs of borreliosis. Spirochetes were demonstrated in the blood, synovial fluid, colostrum and urine by dark-field microscopy and isolations were made by placing 0.1 ml into 7 ml BSK II medium and incubating at 34°C.

One cow with acute laminitis and an IFA antibody titer of 1:1024 to *B. burgdorferi* was reported treated with tetracycline (6 g IV q 12 h) for four days.[38] The clinical condition continued to deteriorate with chronic weight loss, distention of the carpal joints, lameness, and inability to rise without aid. Following euthanasia, gross pathological observations included mottling of the heart and kidneys, excess joint fluid and moderate lymphadenopathy. Histopathological examination revealed evidence of arthritis and synovitis. *Borrelia burgdorferi* was isolated from the lung and liver.

Objectives

The objectives of this research project were to utilize an Indirect Immunofluorescent Assay (IFA) detection technique to evaluate the IgG class immune response and observe for the clinical signs associated with Lyme borreliosis in an experimental bovine model. This was accomplished by challenging gnotobiotic (germfree) calves in environmental isolators with *B. burgdorferi* to eliminate outside biological and environmental factors.

MATERIALS AND METHODS

Animals

The two calves in this study were derived by Caesarean section from Jersey cows serologically negative for antibodies against *B. burgdorferi* and were placed in individual flexible germ free isolators. The calves were derived and maintained by the Gnotobiotic Animal Production Unit at the National Animal Disease Center, Ames, Iowa. Baseline serum samples were obtained from each calf prior to inoculation with *B. burgdorferi* spirochetes (Tables 1 and 2). Spirochete challenges were performed on days two and three following birth using second passage white-footed mouse isolates supplied by Dr. E. C. Burgess of the University of Wisconsin. The inocula was administered via subcutaneous (SC), intramuscular (IM), and intravenous (IV) routes. A routine feeding schedule was maintained (Table 3) and records on diet consumption, temperature, hematocrit, hemoglobin, and general observations were kept. Blood, urine, eye, oral, and fecal samples were taken post challenge for isolation attempts, serology, and blood cell and blood chemistry analyses. By design and isolator restrictions, the animals were scheduled to remain in isolation for 90 days followed by removal to normal animal facilities. The calves were monitored continuously for aerobic and anaerobic bacterial contamination.

The experiment with calf number one was terminated 15 days post inoculation, earlier than expected due to an accident in which the animal escaped from the pen inside the isolator and suffocated in the glove sleeve of the flexible film isolator. The experiment was long enough to permit incubation of the *Borrelia* but inadequate for full serological response.

Calf number two was held in the isolator for 92 days. Two days prior to removal from the isolator, the calf was provided an oral dose of *Lactobacillus acidophilus* and *Streptococcus faecalis* to colonize the intestinal tract and was introduced to unfiltered air by removing the

Table 1. Results of IFA testing for IgG antibodies against Borrelia burgdorferi in the parent of calf number one born 03/23/88.

Sample Date	IgG Titer	Results
02/24/88	< 1:64	Negative
03/23/88	< 1:64	Negative

Table 2. Results of IFA testing for IgG antibodies against *Borrelia burgdorferi* in the parent of calf number two born 05/03/88.

Sample Date	IgG Titer	Results
12/04/88	< 1:64	Negative
05/03/88	< 1:64	Negative

Table 3. Feeding schedule from birth to time of removal from isolation. Surgery day = Day 0. Diet = Similac with Iron, Ross Laboratories, Columbus, OH.

Days 0 - 5	750 ml 2X per day
Days 6 - 7	1080 ml 2X per day
Days 8 - Term	1440 ml 2X per day

HEPA filtration system. The calf was removed on day 93 and eventually weaned off liquid diet. The immune system was suppressed with 2cc Azium Dexamethasone (0.1 mg/kg, IV), Schering Corp., Kenilworth, NJ 07033, on days 136-139 and again challenged with 1.035×10^3 *B. burgdorferi* spirochetes as before. The calf was euthanatized on day 186 and examined at necropsy.

Isolators

Flexible film germ free isolators were equipped as required by the experimental protocol.[61,113] Each unit included High Efficiency Particle Air (HEPA) filters for supply and exhaust air.[137] Sampling equipment and supplies such as syringes, citrated and EDTA blood tubes, serum separation tubes, swabs, and specimen cups were sterilized and placed inside the isolators. The supply of diet (Similac with Iron, Ross Laboratories, Columbus, OH, 43216)) was also included as well as various materials required to maintain the animals during containment in the isolators.

The isolators and contents were sterilized using ethylene-oxide as described by Dennis et al.[61] Following sterilization, a portable sterile germicidal trap containing 10% solution of Wescodyne (West Chemical Products, Inc., New York, NY 10011) was attached.[113]

Procedure

The procedure for cesarean derived germ free calves as described previously was followed.[113] Briefly, pregnant full-term cows were anesthetized with halothane. Cesarean sections were performed using a ventral or flank approach. Aseptic techniques were employed throughout the surgical procedures. The calves were removed from the uterus and passed through the germicidal trap and into the isolator. The calf was then aspirated, ventilated and rubbed vigorously until independent respiration was achieved. The germicidal trap was

removed and the isolator was relocated from the surgery suite to an isolation room. During anaesthesia it was important to administer sufficient halothane to anesthetize the calf to prevent spontaneous respiration before being transferred into the isolator.

Microbial Contamination Monitoring

During isolation, each animal was monitored for aerobic and anaerobic bacterial colonization.[113] Fecal swabs were obtained weekly and cultured for bacteria. Aerobic cultures were performed by incubating swabs overnight in trypticase soy broth at 37°C. Swabs were then streaked onto blood agar plates and incubated at 37°C for 48 hours and checked for growth. Anaerobic cultures were performed by placing swabs into fluid thioglycollate media for transport. Swabs were then transferred under CO_2 to pre-reduced peptone yeast extract broth and incubated at 37°C. Each culture was checked daily for growth.

Inoculum

The inoculum was prepared from a low passage white-footed mouse isolate of *B. burgdorferi* supplied by Dr. E. C. Burgess of the University of Wisconsin. Suitable cultures for the inoculum were established using standard techniques [4,7,84] in Modified BSK medium (Appendix A). Five to seven day cultures of spirochetes were washed 3 times in phosphate buffered saline (PBS) with gentle agitation. Dilutions of PBS inoculum were made and spirochetes were quantified under dark field microscopy using a corpuscle counting chamber. A final concentration of 3×10^3 to 7×10^3 spirochetes per ml was used for challenge. One ml of inoculum was administered at each site (IV, IM, SC) on days two and three post birth. Subcultures of the inoculum remained viable in BSK media three days following its preparation. PBS was chosen for the inoculum suspending fluid to eliminate possible reactions by the host to bovine serum albumin (BSA) Fraction V and other constituents of BSK media.

Samples and Isolation

Multiple samples (urine, blood, oral, eye, fecal) were taken following challenge on a varying schedule for isolation, serology, and blood cell and blood chemistry analysis. Blood cell counts, hematocrit and hemoglobin analyses were performed using electronic methods (Baker Instruments, Hematology Series, Cell Counter 150 and Dilutor 106).

Isolation and culturing of *B. burgdorferi* have been described by others.[4,7,42,43,84] Citrated blood and urine were examined directly under dark field microscopy for the presence of spirochetes. Each blood and urine sample (0.1 ml) was inoculated into 4.5 ml of Modified BSK medium. Ocular and oral swabs were transferred directly into culture media. Suspensions of fecal samples in PBS (0.1 - 0.5 ml) were also inoculated into tubes of media. All cultures were incubated at 34°C and examined weekly under dark field microscopy for the presence of spirochetes.

Pathological samples of heart, lung, liver, spleen, kidney, bladder and synovial fluid collected at post-mortem examination were suspended in .01 M phosphate buffered saline (PBS) pH 7.6 and processed in a Stomacher®. One tenth milliliter of each suspension was inoculated into Modified BSK medium, incubated at 34°C and examined weekly for the presence of spirochetes.

Antibody Detection

Indirect Immunofluorescent Assay (IFA) methods were used to detect presence of IgG antibodies against *B. burgdorferi* [25,35,57,74,109,129] in sera collected in serum separation tubes. Positive and negative control sera supplied by Dr. Burgess were used to assist in the interpretation of results.

Antigen slides for the IFA test procedure were prepared using methods previously described.[25] Briefly, *B. burgdorferi* cultures were grown to mature log phase (5-7 days) in

Modified BSK medium as described previously.[4] Each 4.5 ml tube culture was equally aliquoted into four microcentrifuge tubes and centrifuged for 30 minutes. The resulting pellets of cells were washed in cold PBS w/$MgCl_2$ (1.016 gm/L) with centrifugation for 15 minutes, using two washings. The four pellets were then combined into one tube and washed three more times. After the final wash, cells were suspended in one ml of cold PBS which had been filtered at 0.2 μm. Trial dilutions were made and viewed under dark field microscopy to confirm proper cell concentrations. Filter sterilized PBS was then used to make the final working dilution of cells. The cell suspension was stirred continually to maintain equal concentrations and then placed on printed microscope slides with 8 mm diameter wells (Roboz Surgical Instrument Co., Inc., 1000 Connecticut Ave. N.W., Washington D.C. 20036). One drop (20-30 μl) of cell suspension was added to each well. The antigens were fixed to the slides by drying in a 32°C incubator for 12-24 hours. Prepared slides were stored at -70°C until needed.

Test Procedure

Two-fold dilutions of the serum to be tested for antibodies against *B. burgdorferi* were made with sterile filtered PBS in round bottom microtiter plates beginning at 1:8 and ending at 1:2048. One drop of each dilution was added to individual wells on the antigen slides. Multiple wells allowed for individual samples and controls to be assayed on single slides. The slides with test sera were incubated at 32°C for 45 minutes and rinsed with filtered PBS. Slides were triple washed in PBS in a wash rack with a stir bar for 10 minutes each. Slides were then dipped in filtered distilled water and dried by blotting a single time with bibulous paper. Fluorescein isothiocyanate labeled IgG fraction anti-bovine (heavy and light chain) conjugate produced in rabbits (Organon Teknica Corp. Cappel, One Technology Court, Malvern, PA 19355) prepared in working dilution and centrifuged before use was used as the overlay. One drop (20-30 μl) was placed on each well and incubated at 32°C for 45 minutes. Slides

were washed in PBS, rinsed in distilled water, and dried as before. FA mounting fluid (9:1 glycerol/PBS) was placed in the center painted portions of the slides which were then covered with No.1, 24 x 50 mm glass cover slips (Corning), using slight pressure to remove air pockets. The slides were examined with a fluorescence microscope at low power (16-20x). End points were the highest dilutions to exhibit uniform fluorescence of spirochetes.

RESULTS

Calf Number One

Before calf number one died a total of four serum samples were obtained. Antibodies against *B. burdorferi* were not detected in the serum using IFA methods (Table 4). Post mortem examination revealed normal tissues. Attempts to recover *Borrelia* from the lung, liver, spleen, kidneys, urinary tract, and fluids from the eyes, spinal column, and joints were unsuccessful.

Table 4. IFA results for calf number one. (Spirochete challenge on days one and two)

Sample Day	IgG Titer
0	< 1:8
5	< 1:8
8	< 1:8
12	< 1:8
15	<1:8

Calf Number Two

General observations for clinical signs during isolation in calf number two did not indicate evidence of active infection. Feed consumption was normal and body temperatures fell within a normal range (Figure 1a). White blood cell counts were comparable to other animals raised in the Gnotobiotic Unit (Figure 1b). Aerobic and anaerobic monitoring for bacterial contamination

indicated that the animal was free of unwanted organisms that may have been introduced from outside sources.

Stress to the animal due to the change from a clean environment within the isolator to normal animal facilities resulted in a recorded rise in IgG antibody titer against *B. burgdorferi* (discussed below). In addition, during a 48-hour period following removal from the isolator, the calf was unable to stand and food consumption dropped to zero. Red blood cell counts were also lower when compared to counts observed when the calf was in isolation, (Figure 1c). A typical increase in white blood cell was observed for two days during immunosuppression. The calf was euthanatized on day 186 and examined at necropsy. All tissues examined, as in calf number one, appeared normal with no lesions indicating active infection.

Results of IFA testing (Figure 2) showed that IgG antibodies specific for *B. burgdorferi* began to appear ten days post challenge. Although titers of 1:128 are reported to be diagnostic in naturally occurring Lyme borreliosis, a titer of 1:8 is considered significant in a gnotobiotic animal (Figure 3). Titers rose to 1:256 after 35 days PC and remained in a range of 1:128 to 1:256 for 67 days. Ten days following removal from isolation and environmental stress (day 102 PC), the titer had risen to 1:512. The titer remained at 1:512 throughout periods of experimentally induced immunosuppression and rechallenge. By day 163 PC the titer had dropped to 1:256 and upon termination of the experiment on day 186 PC, the titer was 1:128.

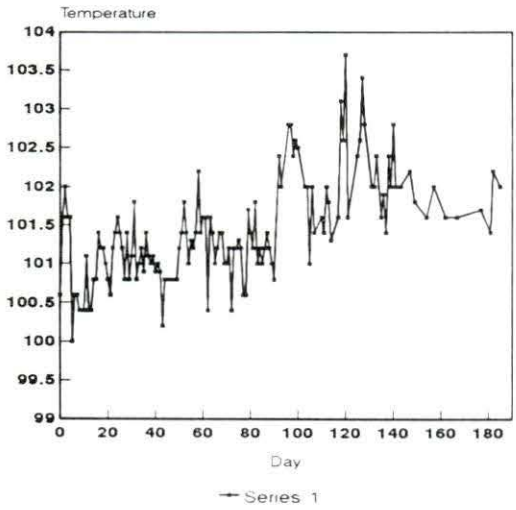
Spirochetes were visualized under dark field microscopy in the first post challenge blood sample, taken ten minutes PC. Blood cultures for isolation of *B. burgdorferi* failed to yield any viable spirochetes. Spirochetes were not isolated from blood, urine, eye swabs, oral swabs, and fecal samples taken during the experiment did not occur. Post-mortem examination did not show any gross lesions of the lungs, liver, spleen, bladder, kidneys, joints, or heart. Culture of

tissues, synovial fluids, and spinal fluid collected during necropsy were negative for spirochetes.

Figure 1. Temperature and Blood Cell Comparisons for calf number two; (A) Temperature fluctuations observed for the duration of the experiment, (B) White blood cell (WBC) counts during gnotobiotic isolation compared to WBC counts of a calf in isolation infected with Bovine Viral Diarrhea (BVD), and (C) comparison of red blood cell (RBC) counts during isolation and after removal to normal animal housing.

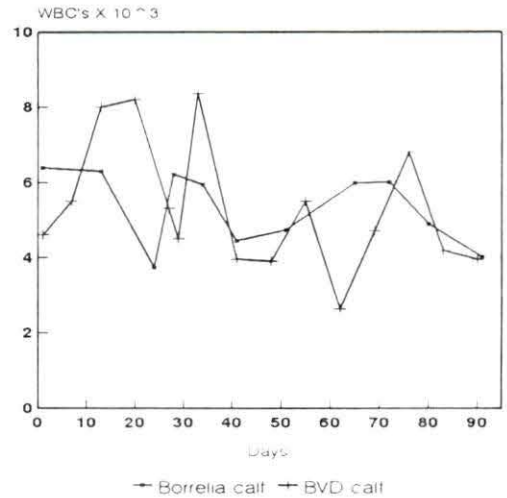
(A)

Body Temperature



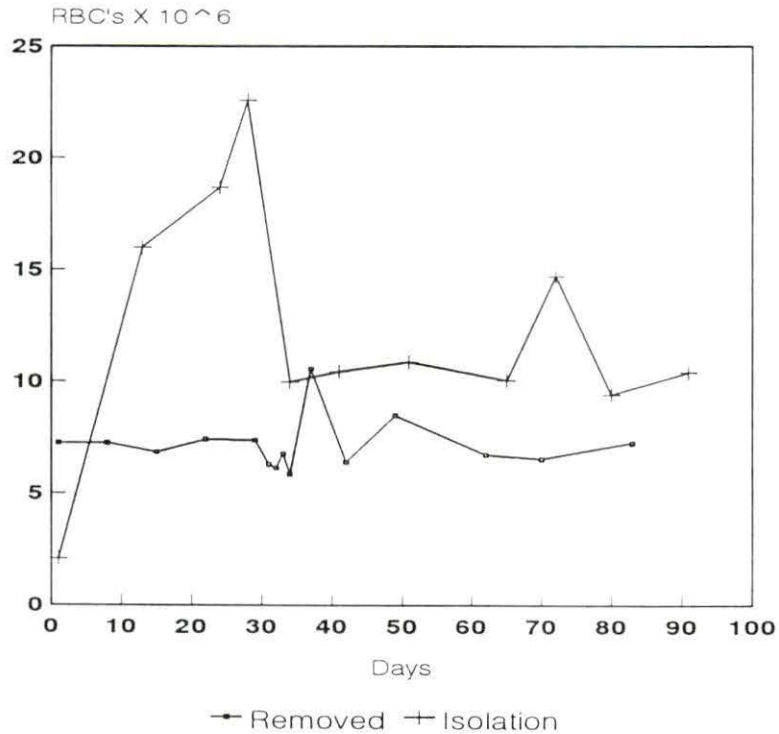
(B)

White Blood Cell Counts
During Isolation



(C)

Compared Red Blood Cell Counts
Removal vs. Isolation



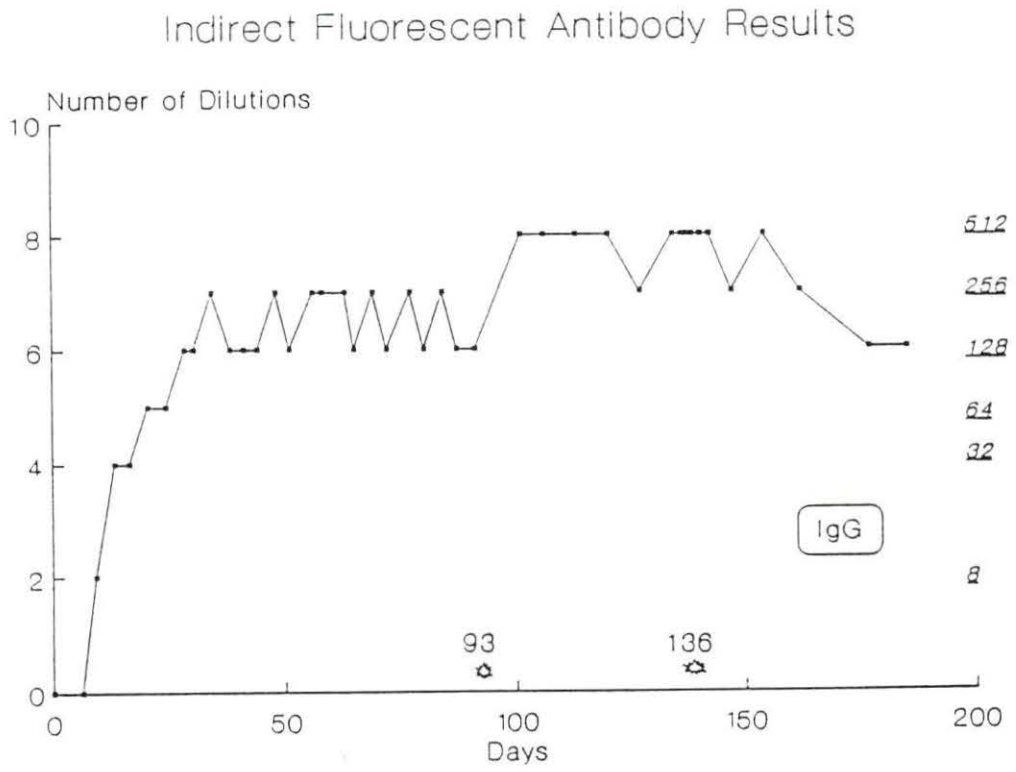
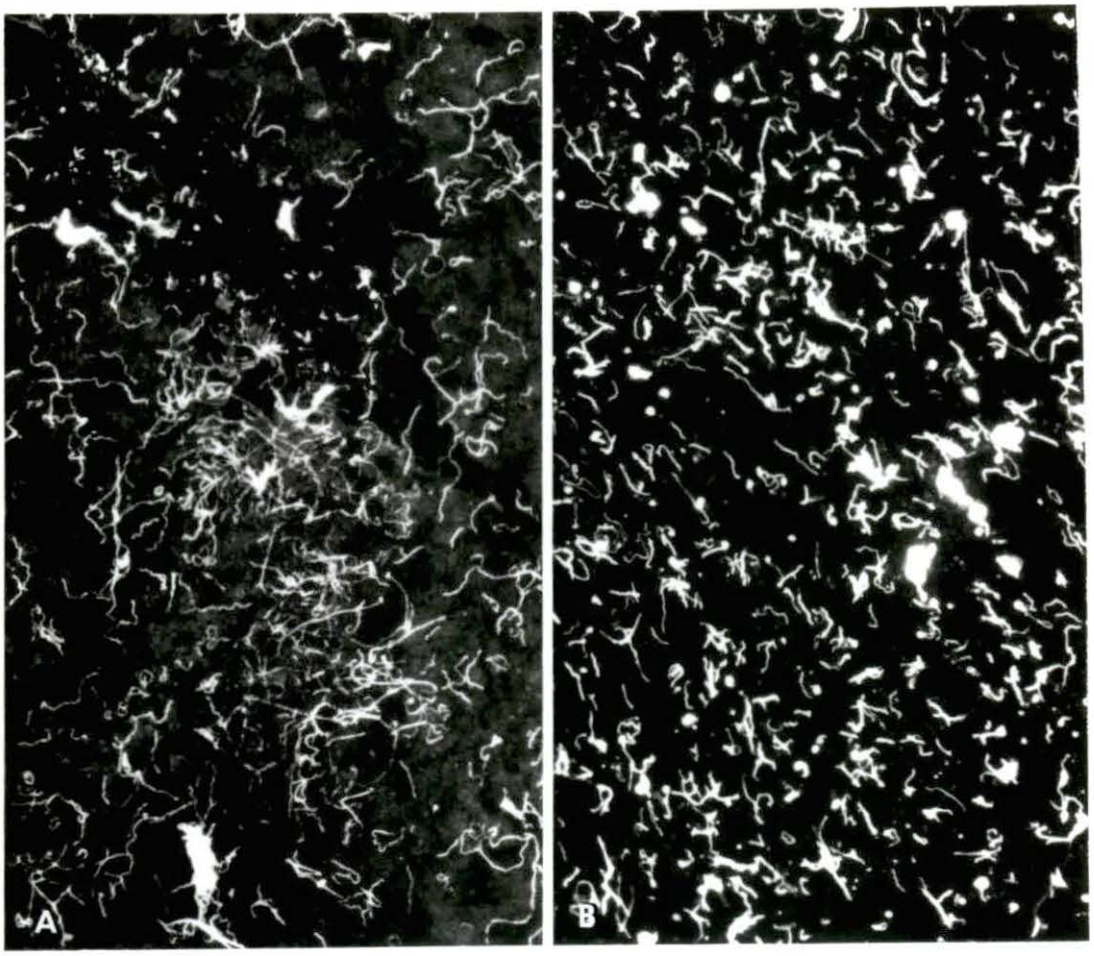


Figure 2. Indirect fluorescent IgG antibody results in calf number two expressed in number of two-fold dilutions over time in days. Antibody response began to appear 10 days post challenge. Day 93 represents day of removal from gnotobiotic isolation and day 136 represents beginning of induced immunosuppression with Dexamethazone and rechallenge.

Figure 3. Comparison of positive IFA tests as viewed under fluorescing microscopy at 20x for; (A) naturally infected cow from Wisconsin and, (B) experimentally infected gnotobiotic calf number two. Note absence of background reaction in the gnotobiotic animal test.


A
B

DISCUSSION

This is the first report of an experimental challenge with *B. burgdorferi* using germ free calves. The expense and logistics required to utilize germ free animals placed strict limitations on the experimental protocol and the number of animals to be included. The lack of apparent clinical disease in the gnotobiotic animal does not indicate that *B. burgdorferi* is not a disease causing agent in cattle.

Removal from gnotobiotic isolation resulted in an apparent rise in antibody titer. This rise in antibody titer was considered to be related to an overall immune response to many bacterial antigens that were not present during isolation.

Immunosuppression and rechallenge with the agent was carried out on the hypothesis that it would increase the magnitude of antibody response and possibly produce clinical manifestations in the animal. There was no significant rise in titer and clinical signs did not become apparent. The pathogenicity of the *B. burgdorferi* organisms used in this study were not established, due to lack of suitable animal models for the disease. The organisms used for the inoculum were of a low passage mouse isolate from an area endemic for Lyme disease. Spirochetes were successfully cultured from the inoculum three days after it was prepared, indicating survival in media other than BSK may be significant. Isolation of the spirochete from the calf had not been anticipated to be difficult, as no competing organisms were expected, however, low numbers of circulating spirochetes was probably a determining factor. Oral, eye, and fecal samples taken while the calf was in isolation remained sterile during incubation periods. Culture from clinically active cases of Lyme disease is difficult from blood in human patients, due in part to the low number of circulating organisms, and laboratory culture for diagnosis is not considered useful.

This project has provided the opportunity to further study and identify the specific host response to a single species of organisms. The IgG immune response was well defined and detection was facilitated without the presence or interference of antibodies to additional organisms. The sera obtained from the gnotobiotic animals while contained in isolation were free of antibodies against antigens other than those developed in response to the challenge organisms. These serum samples will be helpful in future studies for developing diagnostic methods in detecting specific exposure to *B. burgdorferi*. They will be used as absolute positive and negative controls to assist in determining specificity and sensitivity in various assays for antibodies against *B. burgdorferi* and evaluation of antigenic protein constituents of the spirochete.

PART II: EVALUATION OF IMMUNOFLUORESCENCE ANTIBODY (IFA) TEST, WESTERN BLOT, ELISA (FLAGELLA ENRICHED), AND SYNTHETIC POLYPEPTIDE IMMUNOBLOT TECHNIQUES FOR DETECTION OF *Borrelia burgdorferi* EXPOSURE IN BOVINE ANIMALS

INTRODUCTION

The ability to detect evidence of active clinical Lyme Borreliosis in early or late stages, or simply of exposure to the organism, is the subject of intense research efforts. The current assays being utilized by laboratories around the world are not adequate to be used as sole indicators of disease.[138] There is considerable evidence, detailed by studies on antibody detection techniques, that cross-reactivity to other significant disease agents occurs.[51,64,79,104,110,124,147]

Much of the research being conducted today is directed toward finding an adequate antigen marker to be used for detecting specific antibodies against *B. burgdorferi* without the problems associated with cross-reactivity. The problem is very complex due to the nature of the organism. Emphasis has been directed toward isolation of cellular components or individual proteins of the organism which are then characterized for their suitability as antigens.

The most popular antigen of *B. burdorferi* used in immunoassays is the 41 kilodalton (kDa) flagellar protein. It has been used because it is highly antigenic, and is the first protein recognized by the host's immune system.[50,51,54,56,136] Antibodies also persist for longer periods of time in both the IgM and IgG class responses.[58,60,136] The problem in using the flagellar antigen is that there is a certain amount of cross-reactivity with closely related proteins of other organisms, which would make it inadequate as a definitive diagnostic tool.

LITERATURE REVIEW

Laboratory Diagnosis

After the discovery of Lyme disease and the agent responsible for producing disease, it became apparent that there was a broad range of clinical manifestations associated with the disease. The clinical manifestations at presentation can be confused with those of other diseases, making laboratory diagnosis important for proper identification and subsequent treatment of the disease. There are numerous problems associated with serological testing for a disease. Lack of standardization, low sensitivity or specificity, and cross-reactions are the most common and are shared in serological analyses for Lyme borreliosis. The routine methods for detecting antibodies to *B. burgdorferi* and the problems associated with each are discussed in the following section.

Indirect Immunofluorescence Assay (IFA)

The (IFA) test is one of the standard methods for detecting IgG antibodies in the host serum.[36,59,74,93,109,129] In general, it is performed by reacting test serum with *B. burgdorferi* cells that have been fixed on a microscope slide. Following that reaction, fluorescent labeled anti-immunoglobulin is added and resulting reactions are read with the aid of a fluorescing microscope. Although relatively straight forward in concept, it requires highly trained and experienced personnel to carry out the assay. Time requirements are considerable, and the interpretation of end results is subjective. Variation of antigen slides and in visual interpretation result in inconsistencies in results.

Enzyme-Linked Immunosorbent Assay (ELISA)

Another method for detecting antibody levels is the ELISA. This seems to be a better alternative in that it is safer, more sensitive and relatively simple to perform. The assay can be used to determine the type of immunoglobulin produced in an infection, and can measure the

relative degree of immune response. It has been used to detect antibodies in serum, as well as in body fluids such as spinal and joint fluid.[16,19,21,59,74] The ELISA is currently used in many diagnostic laboratories to assist in diagnosing numerous diseases.[44,66] The versatility, ability for automation and rapidity of ELISA assays makes them desirable for diagnostic purposes.

Variations in methods for using ELISA tests mainly lie in the types of antigens used to coat the microtiter wells. These variations alone have resulted in much of the controversy surrounding sensitivity, specificity, and cross-reactions of ELISA protocols. Purified 41 kDa flagellar antigen, the first antigen recognized in antibody production [74,76,78,87,88,89,105] has been shown to have significant homology with other bacterial antigens and as a result, exhibit cross-reactive tendencies. [10,11,52,69,70,71,100,111] Others propose that a crude protein preparation such as sonicated cells makes a better substrate for ELISA testing.[88,89,96,108]

Immunoblot Assay (Western Blot)

This procedure is considered to be an experimental research tool. The procedure is not standardized for Lyme disease testing, is technically complex, time consuming, and requires specialized equipment.[75,76,87,88,99] General methods are to separate *B. burgdorferi* antigens by electrophoresis in acrylamide gel, transfer the protein antigen to a nitrocellulose membrane, react with patient serum, and develop the reaction with enzyme-linked secondary anti-immunoglobulin. As with the ELISA, there are significant findings of cross-reaction with one or more proteins when using Western blotting techniques.[27,82,87] In one study comparing immunoblotting with ELISA, the researchers found 45% of healthy individuals screened by Western blot had IgG antibodies to polypeptides of *B. burgdorferi*, and the majority were to the 41 kDa protein.[76] Hospitalized patients without Lyme disease were tested and many were found to have both IgM and IgG antibodies against *B. burgdorferi*. This is evidence for unrecognized exposure to the agent or significant cross-reactions in tests performed.

Extensive evaluations of the above methods for detecting antibodies to *B. burgdorferi* have been made. These studies have compared specificity and sensitivity between tests and also evaluated different antigen preparations and their effectiveness.[11,88,89] In general, IFA and ELISA testing have shown comparable results, though with some variation. Some studies have indicated a preference for the ELISA due to greater sensitivity and ease of the procedure. Comparisons of results from different laboratories performing Lyme disease testing have detected significant differences, emphasizing the need for standardization.[44,66,93] The Western blot has been considered superior to ELISA and IFA due to greater sensitivity. These studies also point out discrepancies in that the desire for sensitivity in the assays jeopardizes specificity with significant cross-reactivity. In addition, the current methods of detecting antibodies against *B. burgdorferi* work better in identifying immune responses in the late stages of the disease and are less capable of detecting early disease when diagnosis and effective curative treatment are critical.

There are other methods being developed to detect infection by *B. burgdorferi*. Currently their use is limited to a few research oriented laboratories. Two promising methods are the urine antigen test to detect specific antigens secreted in the urine [62,81] and the polymerase chain reaction, (PCR).[72,95,120,126] Detection by PCR using segments of the OspA gene and randomly cloned DNA sequences has been reported. Museum specimens of *I. dammini* ticks have been analyzed for the presence of *B. burgdorferi* DNA using PCR techniques.[119] DNA sequences consistent with today's isolates of *B. burgdorferi* were found in 13 specimens of a 1940s collection from Long Island, New York. Five *D. variabilis* from the same collection were also positive. Other *Ixodes* ticks, from endemic and nonendemic areas were negative.

Flagella and Cross-reactivity

The flagellae of *B. burgdorferi* are located internal to the outer envelope and are composed of a single protein.[10,61,86] Each cell has numerous periplasmic flagellae that can be extracted and purified for analysis and used as an antigen substrate in antibody testing.[16,61,97] The purified periplasmic flagellar protein falls within the region of 39-41 kDa. It is commonly referred to as the 41 kDa protein and is being used extensively as the antigen of choice for enhancing sensitivity in serological testing for Lyme borreliosis. This is the first antigen recognized in both IgM and IgG class antibody responses in patients.[23,68,142] While sensitivity has been achieved, there is abundant information showing that there is significant antibody cross-reactions between the 41 kDa and other flagellar preparations.[54,83,136]

Studies have shown that there is significant amino acid sequence homology among flagellae of other spirochetes[69,97,147] including *Treponema pallidum, Leptospira interrogans*, other *Borrelia*, and oral spirochetes. There is 80% homology of the amino terminus of *B. burgdorferi* flagellin with the 37 kDa subunit of the *T. pallidum* flagellin.[97] In addition, there are several other bacteria which share significant homologous regions within flagellar protein.[69,71,97] The significant amino acid homology with other bacteria may explain the findings of cross-reactivity. A basic anamnestic response to this common antigen by the host could be the basis for apparent dominant, specific, and prolonged antibody response.

The structural gene of *B. burgdorferi* flagellae encodes for a protein of 336 amino acids.[23,70,71] Sequence analysis comparison with flagellae of other related and unrelated bacteria reveals a high degree of conservation. Overlapping octapeptides synthesized and screened using monoclonal and polyclonal antibodies to flagellar proteins to study antigenic properties of the *B. burgdorferi* flagellae have not identified independent immunodominant epitopes however, there is a clustering of reactive oligopeptides within the middle regions of the protein.[23]

In another study, an epitope of the central region (amino acids 205-226) that is heterologous to other bacterial flagellae was found to react well to serum of patients in late stages of Lyme disease.[132] The epitope was not recognized in sera positive for *T. pallidum* and did not react with sera of healthy individuals. This epitope may be a good candidate for use in testing for Lyme borreliosis without complications of cross-reactivity.

Objectives

The purpose of this study is to evaluate IFA, Western Blot, Dot Blot, and ELISA immunodetection techniques to develop an accurate method for determining prevalence of specific exposure to *B. burgdorferi* in bovine animals. Antigen preparations used in the investigation includes enriched and cesium chloride gradient purified flagella, whole cells, and synthetic heterologous polypeptides. Evaluations were by comparison of serum samples from experimentally infected animals to animals naturally exposed to *B. burdorferi*. Sera from normal healthy animals not to be exposed to *B. burdorferi* and animals known to be exposed to other bacterial animal pathogens were also compared.

MATERIALS AND METHODS

Sera

Cow serum samples from Wisconsin were supplied by Dr. E. C. Burgess, University of Wisconsin, Madison, WI. These serum samples were supplied as Indirect Fluorescent Antibody (IFA) test positive for IgG antibodies against *B. burgdorferi*, (Titer of $\geq$ 1:128). Cow serum samples from Iowa were obtained by the Animal Resources Department of the National Animal Disease Center (NADC), Ames, IA. These samples were tested using IFA methods and were negative (< 1:128) for IgG antibodies against *B. burgdorferi*. Iowa is not designated as an endemic area for Lyme disease, although the responsible tick vector, *I. dammini* has been collected and Lyme disease is reported in the state.[117,128] Additional cow sera from animals raised at the NADC with little or no opportunity for tick exposure were used as negative controls. Cattle sera containing antibodies against *L. interrogans* serovars *hardjo* and *pomona*, serum from a rabbit hyper-immunized with flagellar antigens of *T. pallidum*, serum from a pig challenged with *Serpulina hyodysentariae* challenge, and serum from a human infected with *Leptospira* were used in this study. Positive and negative control sera from an experimental challenge of germ free calves with *B. burgdorferi* were also utilized.

Flagella Isolation

The preparation of flagellar antigens is a modification of methods described by Trueba[146] (Figure 4). One to two liters of mature organisms (5-7 days) grown in Modified BSK media were harvested by centrifugation, washed two times in PBS, and suspended in 10 ml of 1% Triton X-114 in 0.1M Tris-HCL (pH 7.8) and 300 μg sodium azide per ml. The cells were incubated for one hour on ice. The outer envelope was partitioned from the cytoplasmic cylinder by centrifugation at 34,000 x g. (15,000 rpm) for 20 minutes.

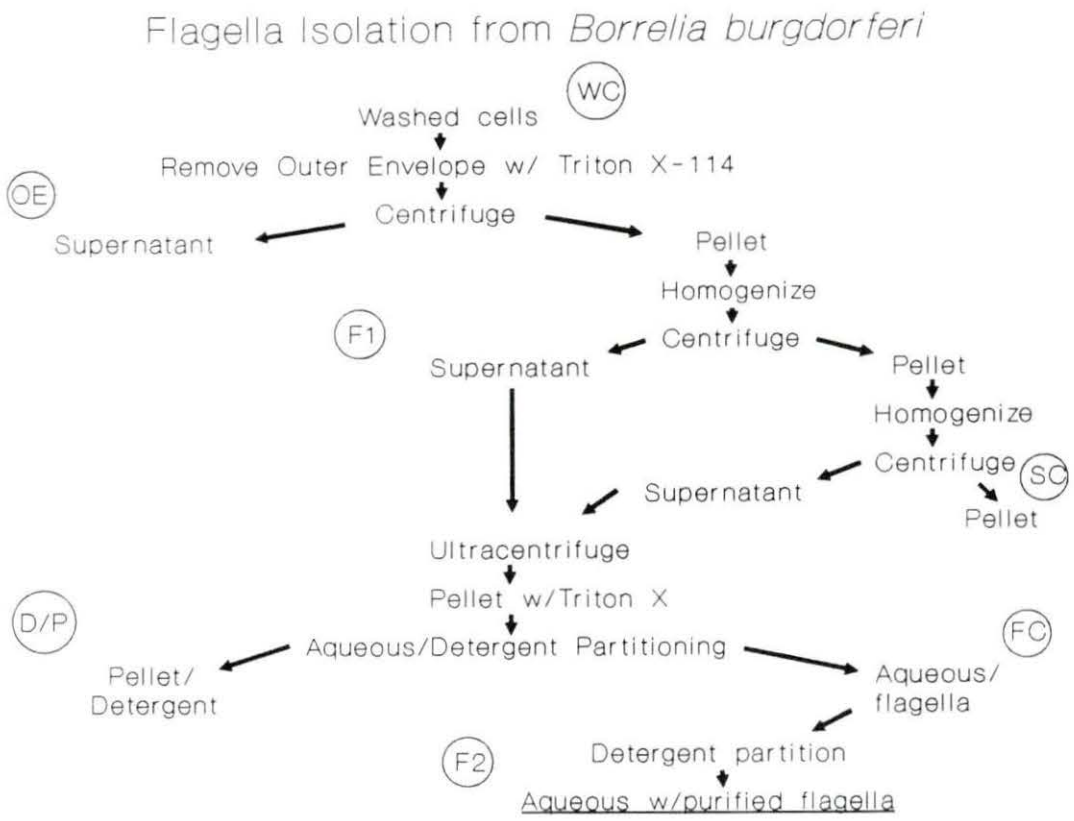


Figure 4. Flow diagram showing enrichment steps for periplasmic flagella isolation from *B. burgdorferi* cells. Major steps are indicated as circled and correspond to lanes of SDS-PAGE analysis in Figure 6.

The supernatant partition containing the outer envelope was frozen at -20°C for SDS-PAGE analysis. The pellet was suspended in 10 ml Tris-Azide and sheared in a Sorvall Omnimixer on the highest setting for 15 minutes on ice. The cellular debris was removed by centrifugation at 34,000 x g. for 20 minutes. The supernatant was saved. The pellet was suspended in Tris-Azide and sheared as before. Debris was removed by centrifugation at 11,000 x g. (9,000 rpm) for 15 minutes and supernatant saved. The preceding two supernatants were pooled and ultracentrifuged at 450,000 x g. (50,000 rpm) for 4 hours at 10°C. The resulting pellets from ultracentrifugation were suspended in 4 ml of 1% Triton X-114, aliquoted into four microfuge tubes, and incubated at 37.5°C for 20 minutes. Following incubation, the tubes were centrifuged for ten minutes in a microcentrifuge. The aqueous partition was saved and extracted once more with 10% Triton X-114 in PBS and centrifuged as before. The final aqueous phase yielded significant concentrations of flagellae as verified by electron microscopy (Figure 5). An aliquot of the final flagella preparation was saved and the remaining preparation was further purified using Cesium Chloride ($CsCl_2$) gradient methods. The final working concentration of $CsCl_2$ was 26%. The gradient, ultracentrifuged at 300,000 g. overnight, revealed three distinct bands. Each band was harvested and dialyzed in distilled water. All steps involved in the above purification procedure were monitored for protein content using SDS-PAGE methods (Figure 6).

Figure 5. Micrograph of the negative stain of the aqueous phase from the final detergent partition step in the periplasmic flagellum isolation protocol. Magnification, 75,400x. Bar = 0.2 μm. The flagella enriched preparation was further purified in a cesium chloride gradient.

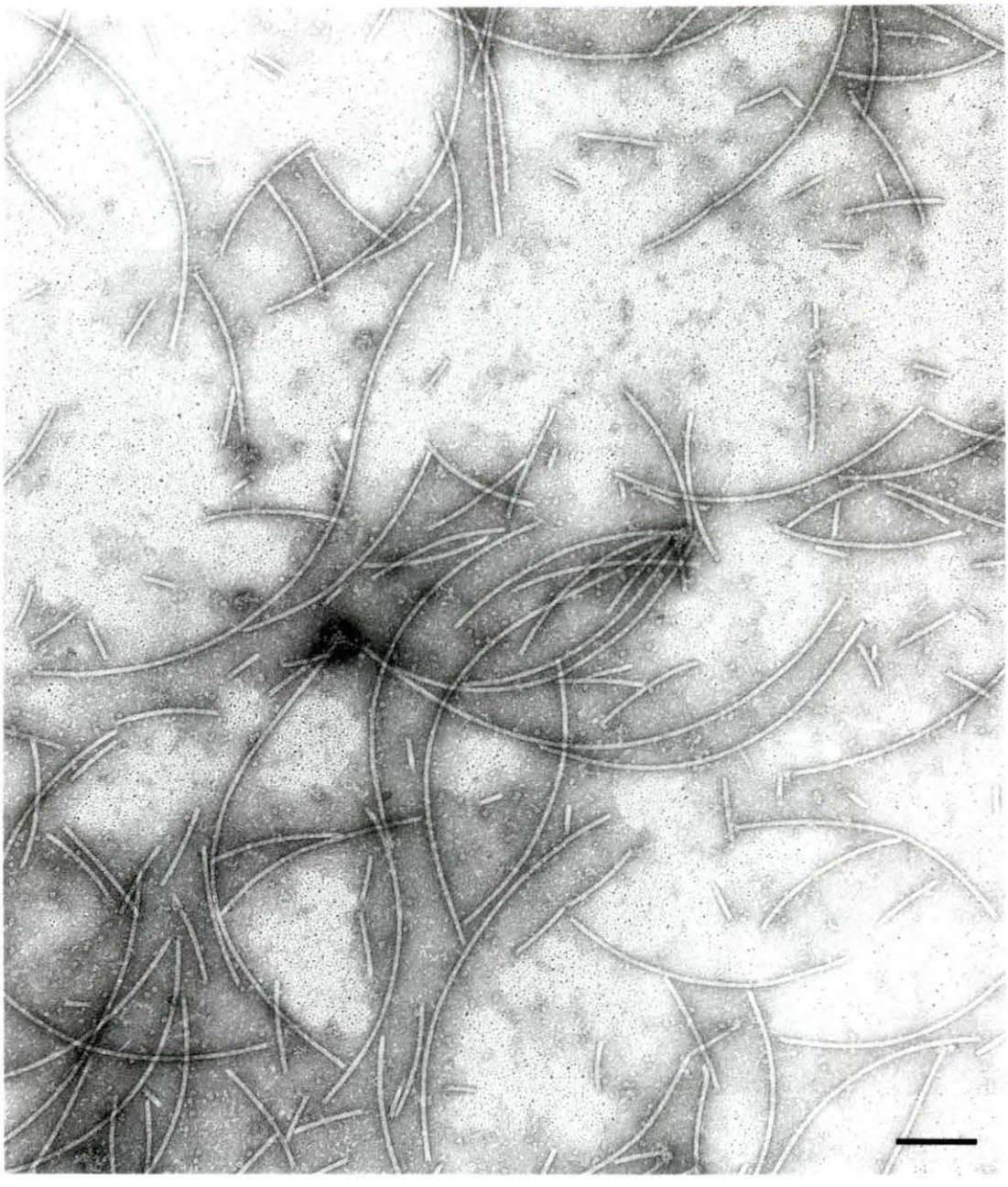
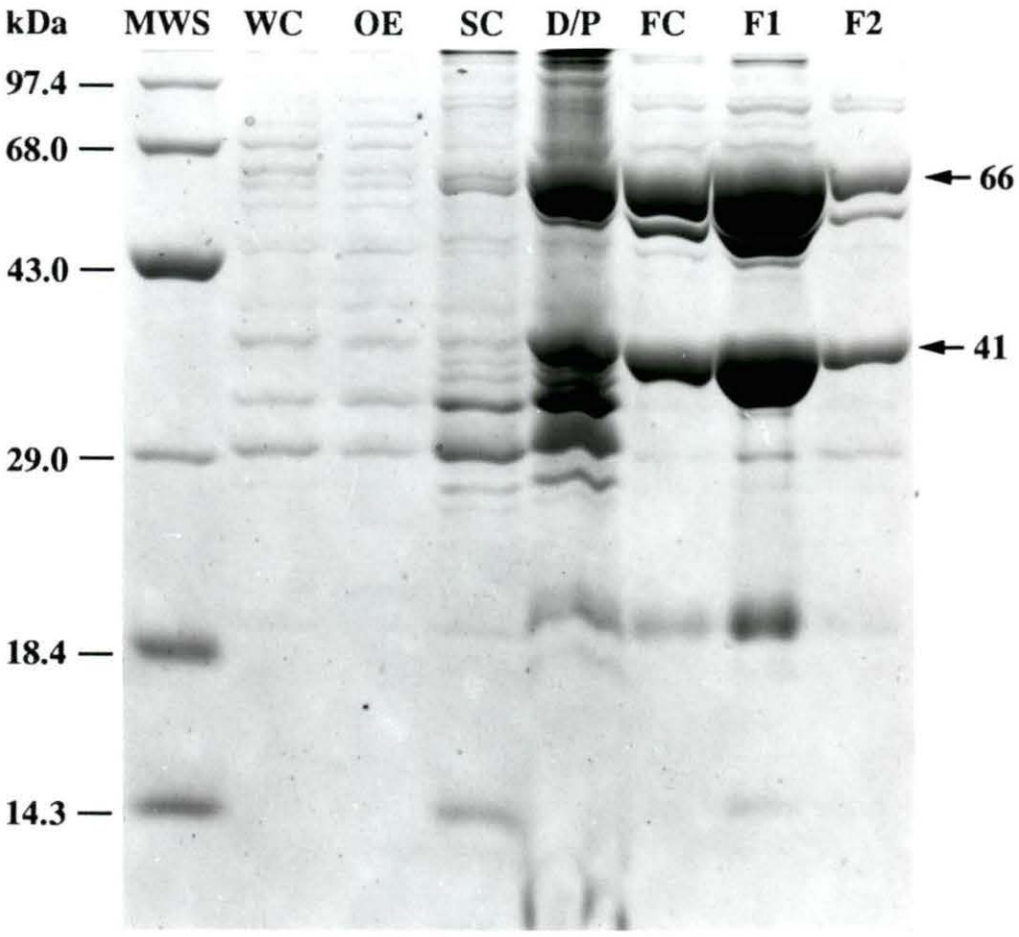


Figure 6.

SDS-polyacrylamide gel electrophoresis protein profiles of *B. burgdorferi* PF extraction steps before cesium chloride purification. Molecular weight standard (MWS) markers were Coomassie Blue myosin H-chain (200 kDa), phosphorylase b (97.4 kDa), Bovine serum albumin (68 kDa), ovoalbumin (43 kDa), carbonic anhydrase (29 kDa), and B-lactoglobulin (18.4 kDa). Lanes correspond to extraction steps (Figure 4) and show the progression to a flagella enriched preparation. WC = whole cells, OE = outer envelope, SC = sheared cells, D/P = detergent phase/pellet, FC = flagella combined, F1 = flagella 1st phase, and F2 = flagella 2nd phase.


kDa
MWS
WC
OE
SC
D/P
FC
F1
F2
97.4
68.0
43.0
29.0
18.4
14.3
66
41

Electron Microscopy

Whole spirochetes and flagellae were stained using 3% Ammonium molybdate. Briefly, the coated side of each prepared, parlodian coated, 200 mesh copper grid was allowed to be in contact with 1 μl of each sample for 45 seconds at room temperature. The excess sample was removed from the grid by blotting the edge of grid to filter paper. The grid was then allowed 5-10 seconds of contact with the 3% Ammonium molybdate. The excess was removed as before. The grids were viewed immediately using a Philips 410 Transmission Electron Microscope (TEM).

Enzyme-Linked Immunoassay (ELISA)

Antigens used for the assay were the flagella enriched fraction prior to the cesium chloride gradient purification step of the flagella extraction protocol detailed above. Dilution factors for antigen (1:2000), conjugate (1:1000), and test sera (1:200) were determined by checkerboard titration. The protocol used has been described previously by Thiermann and Garret [144]. Briefly, 96 well Immulon I plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 250 μl with antigen in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Following 24 hour incubation at 4°C, open binding sites were blocked for 1 hour with 300 μl/well filter sterilized 2% Ovine serum albumin (Sigma Chemical Co., St. Louis, MO) in 0.01 M PBS (pH 7.2) at room temperature. The blocking agent was aspirated and 200 μl of a 1:200 dilution of test serum in 0.01 M PBS (pH 7.2) was added to each well and allowed to incubate for two hours at 37°C. The serum was aspirated and the plates were washed four times with 1% Tween 20 in PBS (pH 7.2). The first wash was completed immediately and the final three were allowed three minutes contact time.

Goat anti-bovine IgG conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) in PBS was prepared at a dilution of 1:1000. Each test

well received 250 µl and plates were incubated for 90 minutes at 37°C. Following incubation, the plates were washed as before. Plates were covered with plastic wrap during all incubation periods to reduce evaporation.

The substrate was prepared by combining 40 ml of 0.96% citric acid buffer (pH 4.0) with 200 µl of 1.83% peroxide solution and lastly adding 200 µl ATBS (2,2-azino-di-[3-ethyl-benzy-thiazoline sulphonic acid]) (USDA, APHIS, National Veterinary Services Laboratories, Ames, IA). Each well received 250 µl of the substrate and allowed to react for 15 minutes at 37°C. The reaction was stopped by adding 50 µl 37 mM solution of sodium cyanide to each well. Reactions were quantified using a Dynatech 3900 EIA reader.

Electrophoresis of Proteins (SDS-PAGE) and Western Blot Immunodetection

Procedures for performing SDS-PAGE and Western blot immunodetection have been previously described.[50,76] Whole cell preparations, proteins from each step of the extraction protocol detailed earlier, and purified flagella were electrophoresed in different runs using standard methods (Bio-Rad). Whole cell preparations were performed using a 200 ml mature culture of *B. burgdorferi* cells. The cells were washed three times in PBS ending with 1 ml washed cells. A total of 700 µl washed cells were added to 400 µl treatment buffer (Appendix B) and 70 µl 2-mercaptoethanol. A Molecular Weight Standard (MWS) kit (SDS-200, Sigma Chemical Co., St. Louis, MO) was used. The SDS-PAGE was carried out using 1.5 mm thick cast gels containing 10% acrylamide under reducing conditions. Each gel was run briefly at 50 mA and lowered to 7 mA for overnight. Once the gel was complete, the stacker portion was trimmed away and the 11.5 x 16.5 cm gel was loaded (LKB, NovaBlot Electrophoresis Kit) for transfer of proteins to Immobilon PVDF membrane. The transfer was run for two hours at 160 mA. Following the transfer, the membrane was removed and allowed to dry. The MWS

portion of the membrane was removed and stained using a coomassie blue solution (Appendix B) for 15 minutes, and destained in destaining solution I (Appendix B) until the desired band color intensities were observed. The gel of the extraction protocol steps was stained in coomsassie blue solution for two hours and destained in Destaining Solution I overnight. Destaining in Solution II (Appendix B) followed for approximately six hours prior to making a photographic record of the gel.

PVDF membrane strips were cut from each run of the whole cell preparation and the purified flagella extraction. Strips were wet with methanol followed by three washings in Tris Buffered Saline(TBS) [Tris 10 mM, 0.9% NaCl, pH 7.4] with 1% Tween 20 for three minutes each. Sera from cows positive and negative for antibodies against *B. burgdorferi* and a person infected with *Leptospira* were diluted to 1:50 in 2 ml TBS with 1% Tween 20 in screw cap tubes large enough to hold one membrane strip. Hyper-immunized rabbit serum for *T. pallidum* was diluted to 1:1000 and serum from a pig infected with *Serpulina hyodysenteriae* was diluted to 1:100. Strips were incubated protein side up in serum at room temperature, rocking for 1.5 hours. The strips were removed and washed as before. A 1:1000 dilution of IgG horseradish peroxidase conjugate in 7 ml TBS with 1% Tween 20 for the bovine and rabbit samples was reacted with the strips for one hour at room temperature. The human and pig antibody conjugates were diluted to 1:500. The strips were removed and washed two times as before followed by two washes in TBS without Tween for three minutes each. A fresh developing solution of 20 ml Methanol (ice cold), 60 mg 4-chloronapthol, 100 ml TBS, and 60 μl 30% hydrogen peroxide was made and reacted with the strips by rocking. Reaction times were determined visually and stopped by placing the strips in distilled water. The strips were dried and displayed for photographic record.

Dot Blot Immunodetection
(Purified flagella and Lipopolysaccharide (LPS))

Antigens used for Dot Blots were purified flagella and LPS extracted from the outer envelope obtained during the flagella isolation steps obtained by methods described earlier. LPS was derived from the outer membrane extraction by simple digestion with pronase.[16 18 77] Each antigen was placed onto a nitrocellulose membrane (2 μl flagella and 5 μl LPS) and allowed to dry. The remaining procedure was followed as described earlier for reaction of strips in the Western blot procedures.

Synthetic Polypeptides (Immunodetection)

The hypothesis for using a synthetic polypeptide from a central non-conserved region of the 41 kDa flagellar protein of *B. burgdorferi* for determining exposure to *B. burgdorferi* in cattle by a rapid dot blot assay was based on a previous study by Schneider et al.[132] Results of that study identified an antigenic epitope within the flagellar gene that is heterologous with amino acid sequences of other bacterial flagellins. The epitope was identified as an amino acid sequence of 20 residues.

The nucleotide sequence for the flagella gene of *B. burdorferi* is known (Appendix C).[71] Synthetic polypeptides from the highly conserved region of the flagellar gene (amino acids 30 - 47) and from the non-conserved central region of the gene (amino acids 207 - 226) were prepared by the Iowa State University Protein Facility, Iowa State University, Ames, Iowa. The purity of each polypeptide was verified using reverse high performance liquid chromatography (HPLC). Preparations of the amino acid sequence from the conserved region were received as a crude product and two peaks harvested from the HPLC purity verification process. The sequence of the non-conserved epitope were also received as a crude form and a single HPLC peak.

Each of the five preparations were used to test serum samples for IgG antibody against *B. burgdorferi* by dot blot immunodetection procedures outlined above. Briefly, polypeptides were dotted onto Zeta-Probe (BioRad), nitrocellulose , or Immobilon PVDF membranes in varying concentrations and allowed to dry. Purified 41 kDa flagella was included on each blot as a control. Alternate binding sites were blocked in TBS with 1% Tween 20 or 1% non-fat milk. Strips were incubated at 25°C rocking for 90 minutes with test serum diluted to 1:50 in TBS with 0.05 Tween 20. Sera tested were from experimental, naturally exposed, and normal bovine animals as detailed earlier. Membranes were washed three times in TBS with 0.05% Tween and reacted with anti-bovine IgG conjugate. Three conjugate systems were used in separate runs to achieve optimum results. They were horseradish peroxidase (1:1000), alkaline phosphatase (1:500), and biotin peroxidase (1:500) / streptavidin (1:1000).[108] (Kirkegaard & Perry Laboratories Inc.) The strips were washed in TBS and reacted with appropriate substrate. Results were determined visually.

RESULTS

ELISA

The results of testing sera from an experimental gnotobiotic calf infected with *B. burgdorferi* and serum from the same animal prior to challenge are shown (Figure 7a). The data indicate the ELISA methods distinguish between positive and negative samples. Differentiation between positive and negative animals is also seen using a naturally infected cow form Wisconsin (IFA 1:512) compared to the germ free calf as a negative control (Figure 7b). When the naturally infected animal from Wisconsin and a negative normal cow from Iowa, (IFA $<1:128$) are compared using the ELISA system (Figure 7c), the ability for the EIA system to distinguish between positive and negative samples is not evident. The same is indicated using a negative cow born and raised at the NADC with no opportunity for tick exposure (Figure 7d). These results and observations are not satisfactory for determining exposure to *B. burgdorferi* in prevalence studies and give support to cross reactive factors to certain antigens of the organisms.

SDS-PAGE

The pattern of the Coomassie blue stained single dimension gel was consistent with previously observed results with runs of whole cell preparations of *B. burgdorferi*.[50] Electrophoresis of protein extracts from flagella enrichment procedures, detailed earlier (Figure 6), shows the progression of each partition step. The presence of purified 41 kDa flagellar proteins is confirmed in SDS-PAGE results of runs from three bands of cesium chloride density gradients of the flagella enriched preparation (Figure 8).

Figure 7.

ELISA results (Solid Black Bars) using the 41 kDa flagella enriched antigen comparing; (A) experimentally infected calf pre and post challenge to *B. burgdorferi* with subsequent elevated IFA IgG titer, (B) a germ free calf and an IFA positive naturally exposed cow from Wisconsin, (C) an IFA positive cow from Wisconsin and an IFA negative cow from Iowa, (D) an IFA positive cow from Wisconsin to an IFA negative cow raised at the NADC.

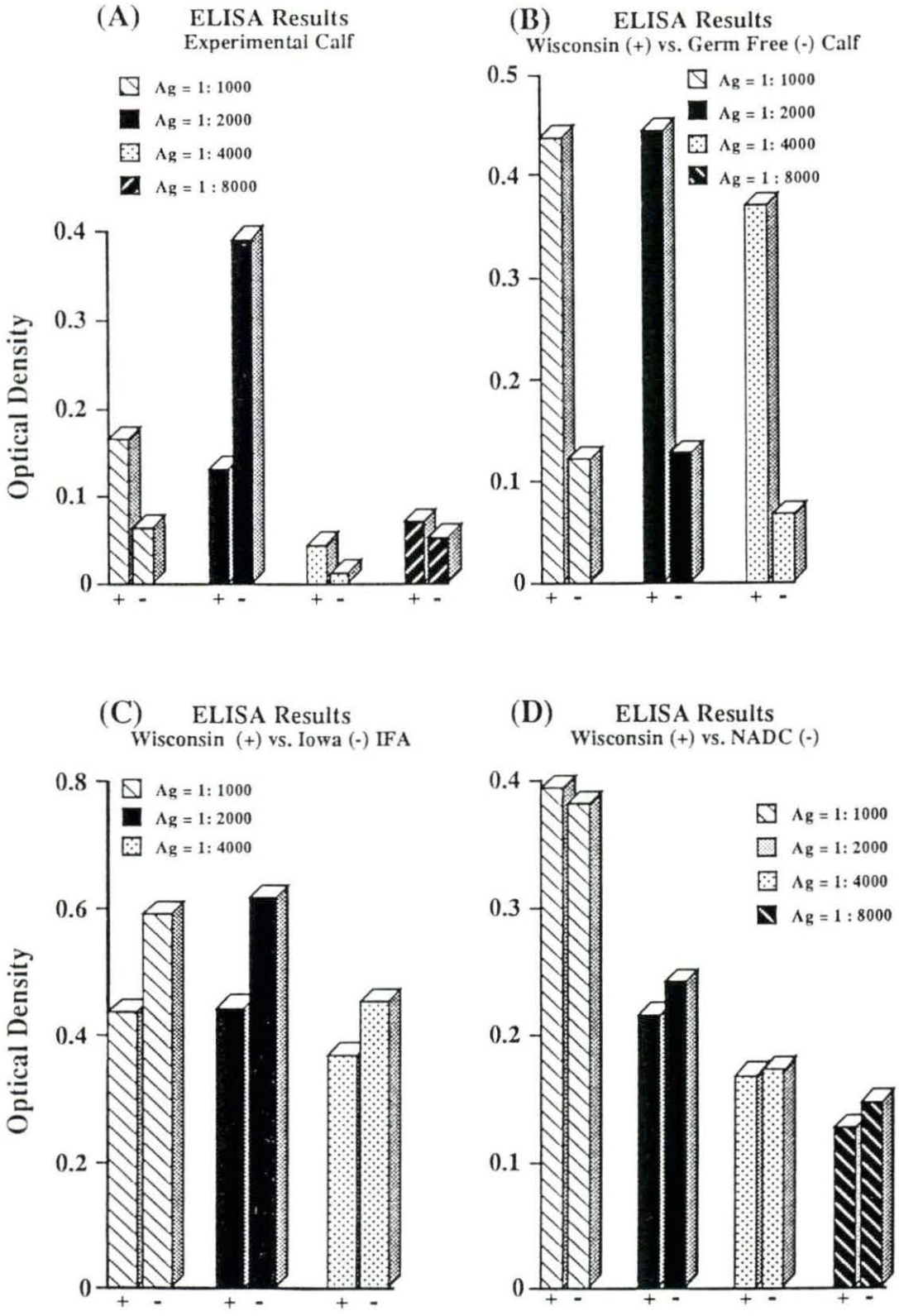
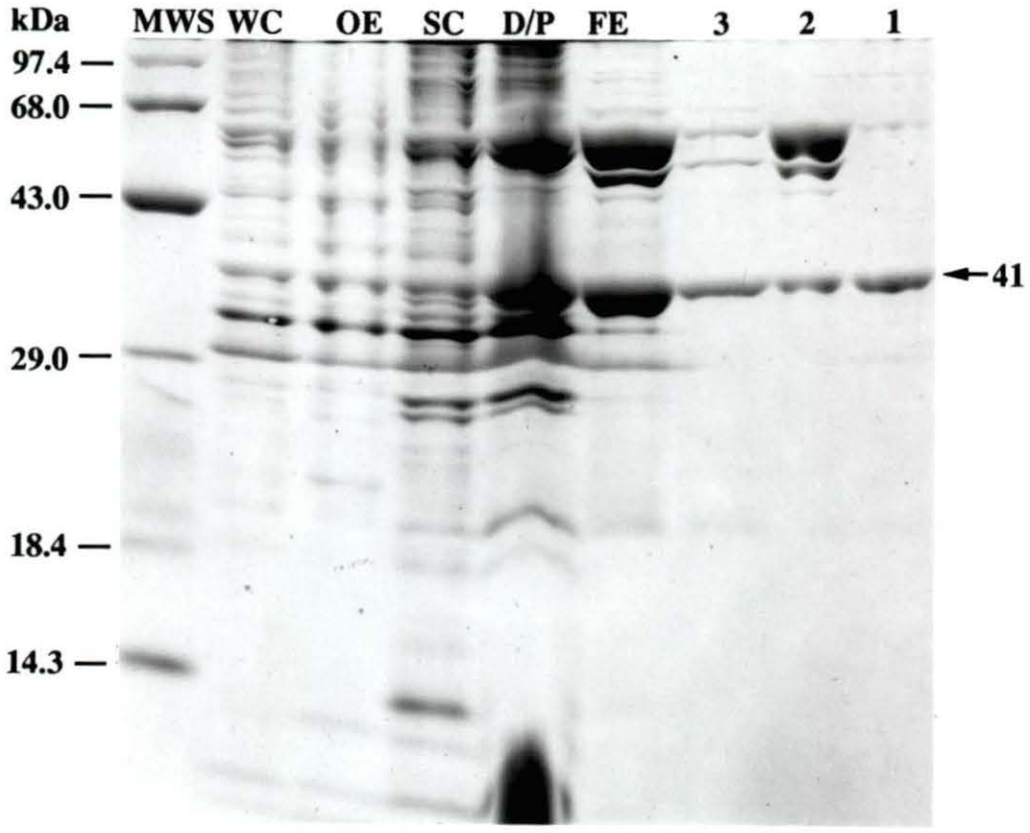

(A) ELISA Results
Experimental Calf
Ag = 1: 1000
Ag = 1: 2000
Ag = 1: 4000
Ag = 1 : 8000
Optical Density
0.4
0.3
0.2
0.1
0
+ - + - + - + -
(B) ELISA Results
Wisconsin (+) vs. Germ Free (-) Calf
Ag = 1: 1000
Ag = 1: 2000
Ag = 1: 4000
Ag = 1 : 8000
0.5
0.4
0.3
0.2
0.1
0
+ - + - + -
(C) ELISA Results
Wisconsin (+) vs. Iowa (-) IFA
Ag = 1: 1000
Ag = 1: 2000
Ag = 1: 4000
Optical Density
0.8
0.6
0.4
0.2
0
+ - + - + -
(D) ELISA Results
Wisconsin (+) vs. NADC (-)
Ag = 1: 1000
Ag = 1: 2000
Ag = 1: 4000
Ag = 1 : 8000
0.4
0.3
0.2
0.1
0
+ - + - + - + -

Figure 8. SDS-PAGE protein profiles showing progression of periplasmic flagellum isolation from *B. burgdorferi*. Lanes 3, 2, and 1 represent the three cesium chloride gradient bands.


kDa
MWS
WC
OE
SC
D/P
FE
3
2
1
97.4
68.0
43.0
29.0
18.4
14.3
41

Western Blot (Whole Cell)

Western blot analysis for IgG antibodies binding to polypeptides of *B. burgdorferi* in serum from an experimentally infected gnotobiotic calf, normal animals from Iowa, and animals naturally exposed from endemic Wisconsin, as determined by IFA testing revealed little variation in band patterns (Figure 9). The contrast intensities for the patterns associated with the experimental animal are apparently less than those for normal or naturally exposed animal cows; however, progression of IgG antibody formation against the organism over time is evident. Significant differences were not observed in the patterns associated with the normal cows from Iowa and those of IFA (+) Wisconsin animals. Numerous major proteins [31-, 34-, and 41 kDa (flagellar)] are represented in each sample tested. The animals representing the Iowa group were IFA negative with titers less than 1:64 except for lane number 8 which was positive at 1:256. In contrast, the Wisconsin samples were IFA positive with titers of 1:128 or higher except for lane number 18 which was negative at 1:64. These results showed that use of whole cell preparations for immunodetection by Western blot is not useful for determining exposure to naturally occurring Lyme borreliosis.

Western Blot (Purified Flagella)

Results of Western blot analysis for IgG class antibodies against the purified 41 kDa flagellar antigen of *B. burgdorferi* revealed strong reactions in the animal species tested (Figure 10). Reaction occurred in sera of an animal experimentally infected with the organism, a naturally infected animal as determined by IFA, two cows infected with *Leptospira pomona* and *hardjo*, a

Figure 9. Western blot analysis and comparison for IgG antibodies against *B. burgdorferi* in serum from; Lanes 1-5, an experimentally infected gnotobiotic calf; Lanes 6-11, bovine sera from non-endemic Iowa; Lanes 12-21, bovine sera from endemic Wisconsin. All samples from Iowa were IFA negative < 1:128) and samples from Wisconsin were IFA positive (>/= 1:128). Lane number one is from a germ free calf prior to challenge with *B. burgdorferi* and lanes 2-5 are 12, 32, 104, and 170 days post inoculation. Lanes four and five also represent samples taken following removal from gnotobiotic isolation.

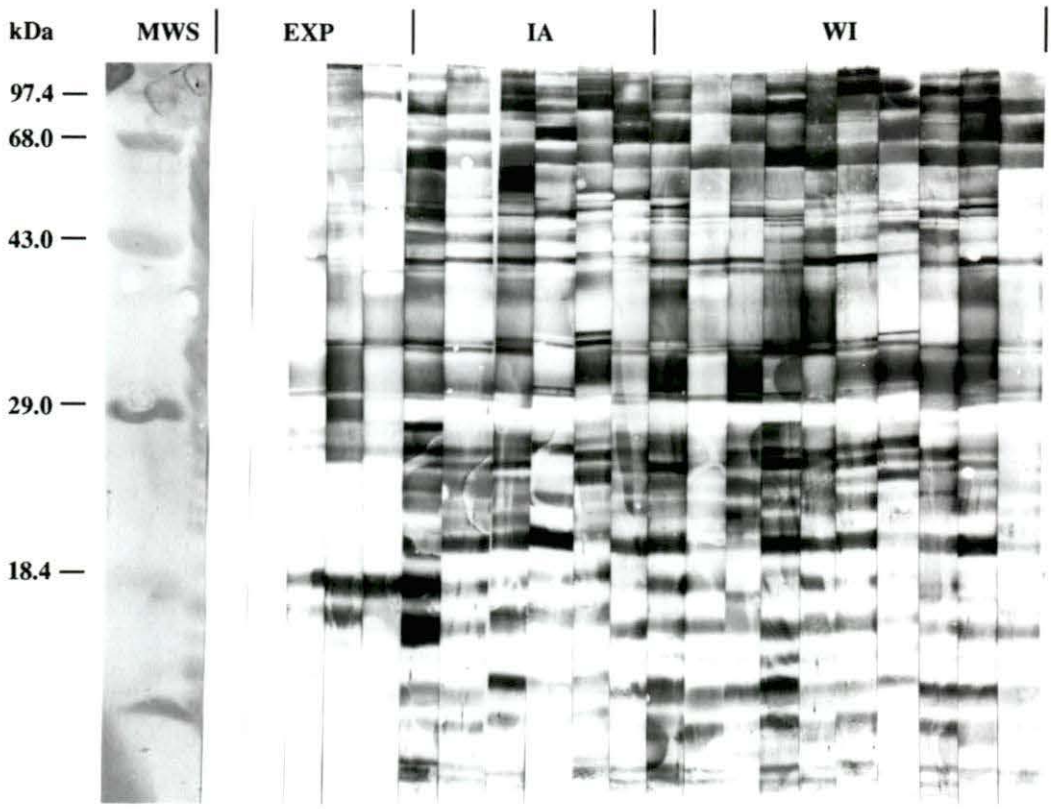
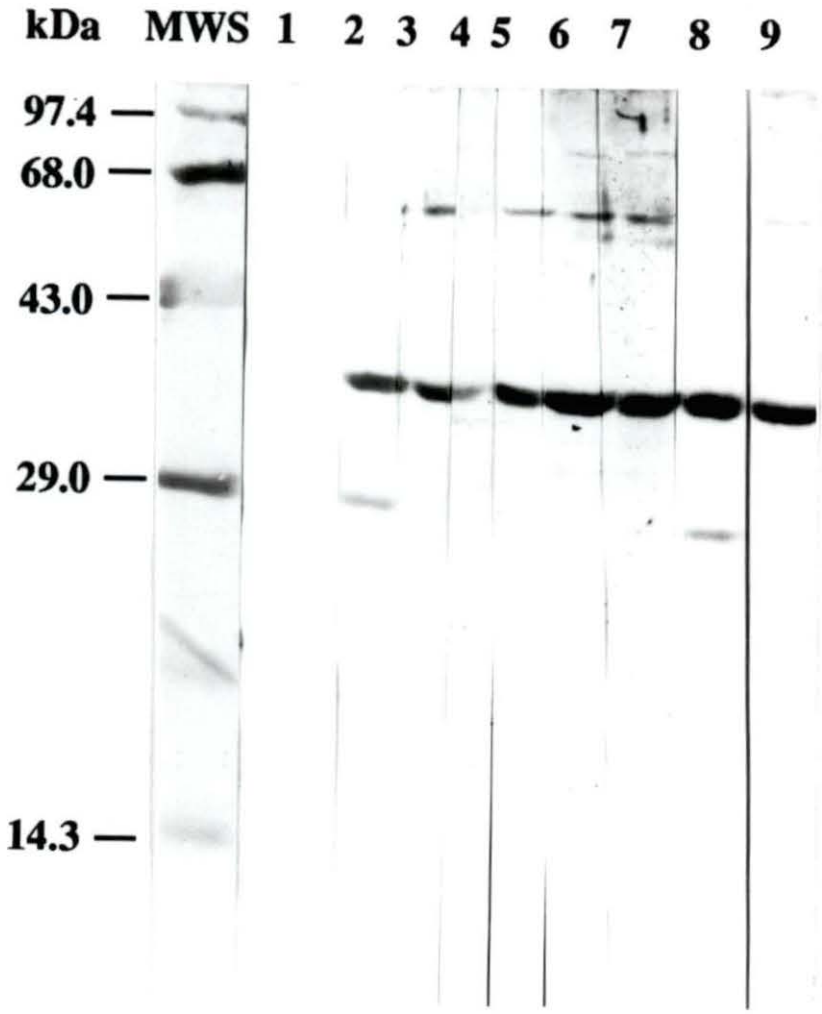

kDa
MWS
EXP
IA
WI
97.4
68.0
43.0
29.0
18.4

Figure 10. Western blot immunodetection of IgG antibodies against cesium chloride purified flagellar protein of *B. burgdorferi* in multiple species infected with *B. burgdorferi*, *Leptospira interrogans* serovar *hardjo* and *pomona*, *Treponema pallidum*, *Serpulina hyodysenteriae*, and a cow with no tick exposure. Lanes: (1) Germ free with no exposure to biological agents, (2) Gnotobiotic calf 60 days post challenge with *B. burgdorferi*, (3) cow from the NADC with no tick exposure, (4) IFA positive (1:512) for Lyme borreliosis cow from Wisconsin, (5) cow infected with *Leptospira pomona*, (6) Cow infected with *L. hardjo*, (7) human being infected with *Leptospira*, (8) rabbit exposed to *Treponema pallidum*, and (9) pig infected with *Serpulina hyodysenteriae*.


kDa
MWS
1
2
3
4
5
6
7
8
9
97.4
68.0
43.0
29.0
14.3

normal animal from the NADC with no opportunity for tick exposure, a rabbit hyperimmunized with *T. pallidum*, a person infected with *Leptospira* acquired through a laboratory infection, and a pig experimentally infected with *S. hyodysentariae*. Serum from a germ free calf prior to challenge with *B. burgdorferi* was the only sample which failed to react with the 41 kDa protein.

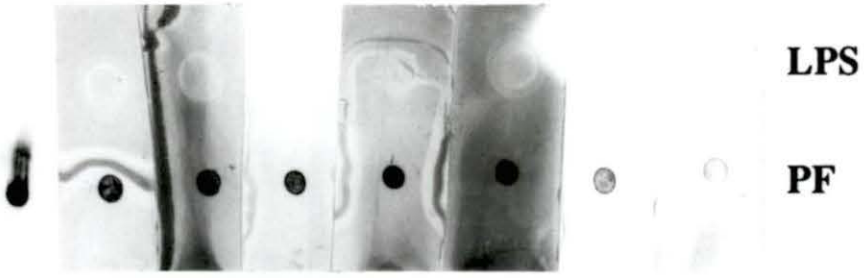
The cesium chloride density gradient purified preparation contained small quantities of protein constituents approximately in the 66- and 31 kDa range. Some samples tested did show reaction in the bands; however, the significance of these reactions was not investigated.

The results of the WB test using a purified 41 kDa flagellar protein gives further confirmation of cross-reactive properties with other bacterial antigenic components. The highly antigenic characteristics of the protein leads to superior sensitivity, however specificity is sacrificed due to the observed cross-reactive tendencies. As indicated in the reported ELISA results, the flagella may not be acceptable as an antigen for use in determining exposure rates in a population.

Dot Blot

Immunodetection results using rapid dot blot procedures were consistent with those of the Western blot analysis detailed above (Figure 11). The same serum sample set that was used for the previously discussed Western blot assay was used for the dot blot, and all samples, except for germ-free samples, reacted to the flagella antigen. Antibodies against Lipopolysaccharide (LPS) extractions could not be detected in any sample. Concentrations of LPS were not determined. The LPS may not be adequate for detecting exposure to the Lyme borreliosis agent in cattle using dot blot methods.

Figure 11. Immunodetection by dot blot technique for IgG antibodies against purified flagella and LPS of *B. burgdorferi* in multiple species infected with *B. burgdorferi*, *Leptospira interrogans* serovar *hardjo* and *pomona*, *Treponema pallidum*, *Serpulina hyodysenteriae*, and a cow with no tick exposure. Lanes: (1) Germ free with no exposure to biological agents, (2) Gnotobiotic calf 60 days post challenge with *B. burgdorferi*, (3) cow from the NADC with no tick exposure, (4) IFA positive (1:512) for Lyme borreliosis cow from Wisconsin, (5) cow infected with *Leptospira pomona*, (6) Cow infected with *L. hardjo*, (7) human being infected with *Leptospira*, (8) rabbit exposed to *Treponema pallidum*, and (9) pig infected with *Serpulina hyodysenteriae*.


LPS
PF
1
2
3
4
5
6
7
8
9

Synthetic Polypeptide Immunodetection

Computer analysis for hydrophobicity and sequence homology characteristics shows significant hydrophobic regions in the non-conserved and conserved polypeptides. Homology is lacking in the central region of the gene but is evident in the 3' region.

The results of immunodetection using the selected synthetic polypeptide and procedures indicate that it is not adequate for detection of antibodies against *B. burgdorferi* in bovine animals. Expected cross-reactive results using the amino acid sequence of the conserved region were not observed. IgG antibodies, known to be present in experimental animals, were not detected using the dot blot assay with the non-conserved epitope. Naturally exposed and normal bovine sera also did not react.

DISCUSSION

The earliest detectable antibodies formed by the host in Lyme borreliosis are of the IgM class and are directed against the flagellar protein with a molecular weight of 41 kDa.[51,52,56] Later, IgG antibodies are also formed against the immunodominant antigen.[58,136] The response to the genus-specific flagellin persists for long periods and provides little, if any, immunological protective value.[66]

Previous evidence indicates that the endoflagellae of *B. burgdorferi* are totally enclosed in the periplasmic space between the protoplasmic cylinder and the outer envelope making the flagellae inaccessible for immune recognition.[80,85] The fact that the earliest detectable immune response is directed towards the flagellae suggests that exposure of the antigen to the surface must occur at some point during infection by disruption of the outer envelope or by other means of surface exposure. The ease of outer envelope disruption was demonstrated when making cell preparations for electron microscopy (Figures 12, 13, 14). Gentle physical action associated with washing spirochetes was important for obtaining intact cells suitable for electron micrographs. It is not unlikely that a certain number of cells may become damaged during transmission to the host from the tick vector. The cell mediated immune response process could also account for liberation of the otherwise concealed flagellar antigen.

There have been numerous reports of significant amino acid sequence homology of the *B. burgdorferi* flagellar protein with other bacterial flagellins.[69,70,71,97,147] Comparisons with *T. pallidum, Bacillus subtilis*, and other *Borrelia* species demonstrate significant homology either at the NH_2-terminus or carboxy-terminus regions, and in the case of other *Borrelia* species, throughout the sequence.

Figure 12. Micrograph of the negative stain of the cell terminus of *B. burgdorferi* showing; outer envelope (OE), cytoplasmic membrane (CM), flagella insertion points (IP), and discs (D) of the flagellae (F). Magnification, 140,400x. Bar = 0.1μm.

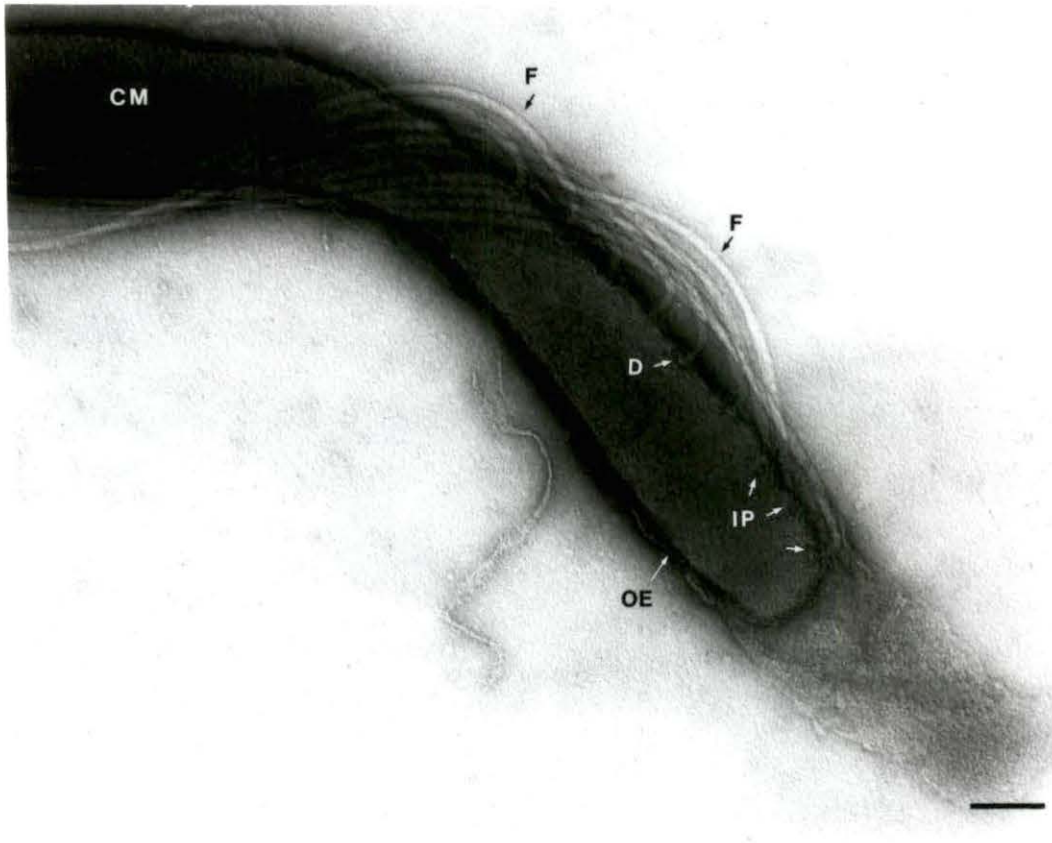

CM
F
F
D
IP
OE

Figure 13. Micrograph of the negative stain of *B. burgdorferi* showing exposed flagellum (F). Magnification, 14,040x. Bar = 1μm.

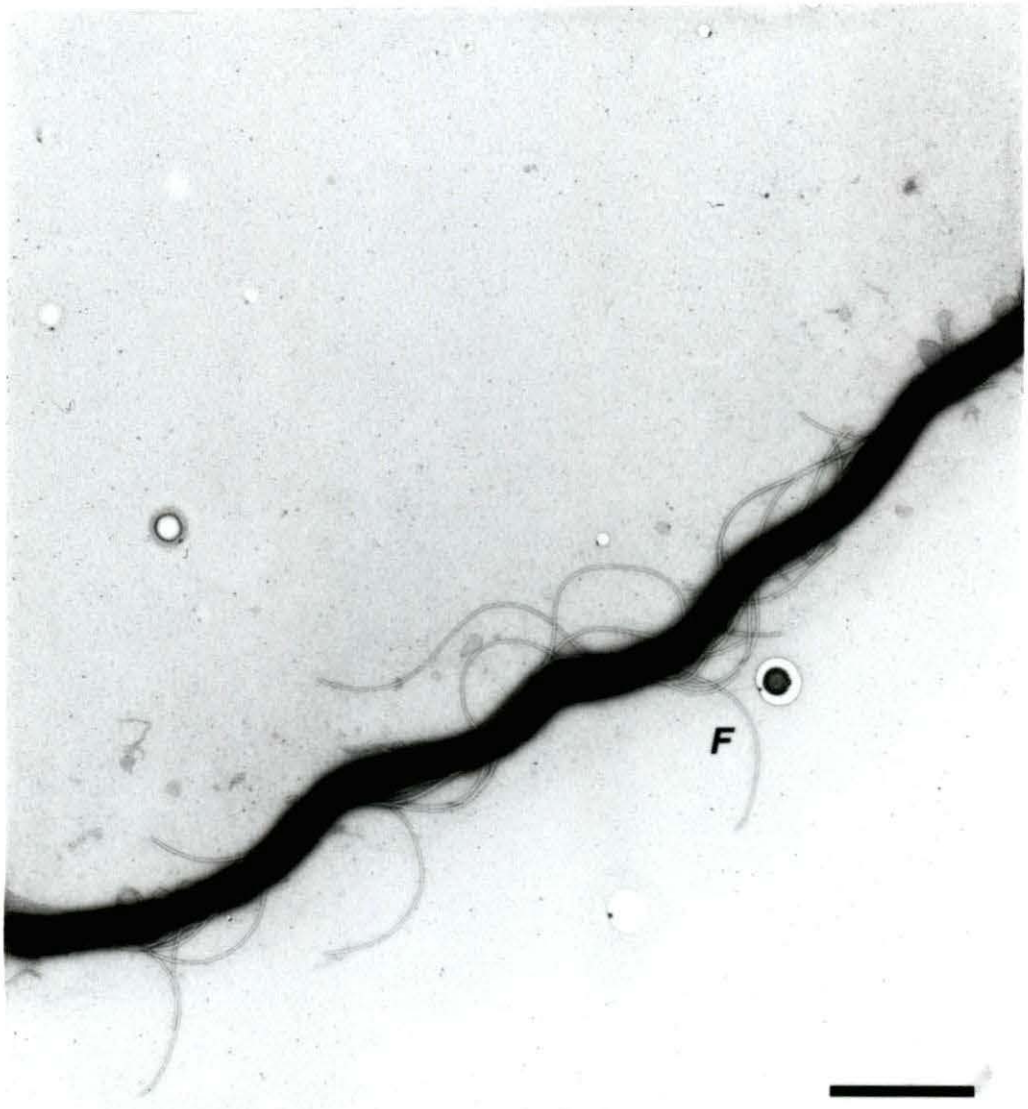
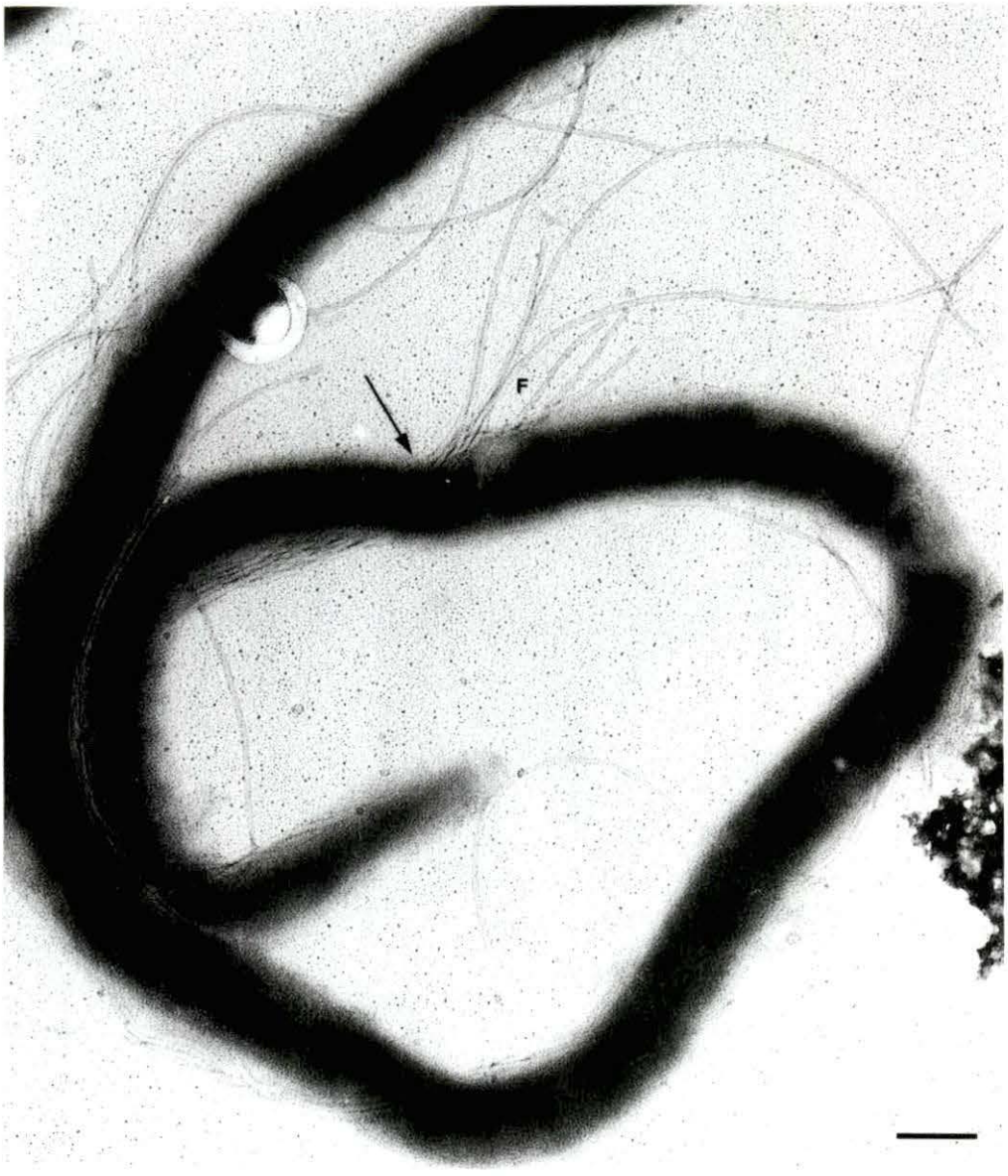

F

Figure 14. Negative stain micrograph showing disruption of the outer envelope (arrow) and periplasmic flagella (F) liberation in *B. burgdorferi*. Magnification, 75,400x. Bar = 0.2μm.


F

In light of the observed cross-reactive nature and amino acid sequence homology, the early emergence and persistent duration of sustained IgG response in the host may be due to an anamnestic response. As a result of the host's immune system recognizing a particular antigen, such as the 41 kDa protein, as one to which it has previously responded, the response would be rapid and of large proportions. The ensuing antibody formation could therefore have been interpreted as specific to the flagellar antigen, creating the idea that it is an immunodominant protein and suitable for diagnostic assays. The recognition of other antigenic constituents of the *Borrelia* organism may indeed occur sooner than those of the 41 kDa protein and may be more specific for the organism but may be masked by the overwhelming flagellar response.

Investigation into the use of an antigenically recognized non-conserved epitope in the central region of the flagellar gene of *B. burgdorferi* identified by Schneider et al [132] did not provide evidence that a synthetic peptide (residues 207 - 226) could be used for determining exposure to the agent in cattle. In addition, results did not give evidence that amino acids 30 - 47 synthetically produced from the leading portion of the flagella protein were independently responsible for cross-reactivity.

The results of this study demonstrate the significant cross-reactive nature of the endoflagellar protein of *B. burgdorferi*. Use of the 41 kDa protein in enriched or purified preparation was shown to be ineffective for use in detecting specific immune response to the *B. burdorferi* in cows. Differences between naturally exposed cattle from Wisconsin and normal animals with no opportunity for tick exposure could not be determined using methods described in Western Blotting, ELISA, or Dot Blot procedures. The limiting factor was assessed to be cross-reactivity. The sensitivity associated with the use of the flagella is exceptional but may be related to the cross-reactive nature of the protein, thereby sacrificing specificity. In contrast, IFA methods appear to provide the most accurate means to test for exposure rates to

B. burgdorferi in a population of cattle. There is little evidence for cross-reaction using IFA methods.

The need for a rapid and accurate method for determining prevalence of the Lyme borreliosis agent in a geographical location is needed. IFA testing is time consuming, difficult to quantitate, and highly subjective. The use of controls is suggested for comparison for each test. Further investigation is required in the area of specific antigens of the *B. burgdorferi* organism to be used for definitive assessment of exposure to the agent. The ability to make early detection of exposure and subsequent active infection will aid in the prevention, treatment, and overall management of Lyme borreliosis.

GENERAL SUMMARY

The results obtained in Part I show that a distinct response to the agent occurs in calves. Clinical presentations of active infection was not observed. The initial response to the organism may have been sufficient to eliminate the development of disease. The virulence of the isolate used, although of low passage, was not determined due to the lack of an accepted animal model. IgG class antibodies persisted at elevated detectable levels for a long period. The antibody levels increased following re-challenge of the animal due, in part, to a basic anamnestic response by the host to a recognized antigen.

Serum samples from the experimental animal of Part I, naturally exposed IFA positive cows from Wisconsin, IFA negative cows from Iowa, cows infected with *Leptospira*, normal cows from the NADC with no potential for exposure to ticks, a rabbit hyper-immunized with *T. pallidum* flagella, a human being exposed to *Leptospira*, and a pig infected with *S. hyodysentariae* were used in Part II of this study.

Positive and negative samples from the experimental animal of Part I could be distinguished in all assays except those using the synthetic polypeptide. Differences could not be determined when comparing naturally exposed and normal animals with any assay except for IFA methods. Samples not infected with *B. burgdorferi* reacted strongly to flagellar antigens indicating cross-reactivity with other organisms such as; *Leptospira*, *T. pallidum* and *S. hyodysenteriae*, or other nonpathogenic agents.

In summary, these studies have provided insights to antibody response in bovine animals and have demonstrated, as others, that there are significant cross-reactive characteristics associated with antigens of *B. burgdorferi*. IFA test methods are currently the most accurate standardized approach available for detecting specific antibodies against Lyme borreliosis in cattle. Further investigations are needed to identify an ideal antigen which can be used in

rapid, accurate testing for Lyme borreliosis in animals as well as human beings. Early diagnosis and effective treatment are keys for management of this disease.

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ACKNOWLEDGEMENTS

I wish to sincerely thank my friends and co-workers at the National Animal Disease Center and Iowa State University for their assistance and encouragement. Special recognition goes to Dr. George W. Beran, Dr. Carole A. Bolin and Dr. Gabriel A. Trueba for their support, guidance, attention and patience.

APPENDIX A
BSK MEDIA PREPARATION

1. All glassware must be rinsed with ultra stilled water. Use autoclaved and .22μm filtered ultra stilled water throughout procedure.

2. Prepare reference buffer of 1N NaOH. Store in refrigerator but use at room temperature.

3. Add 50 ml of water and 2.5 gm Neopeptone. Heat while swirling until dissolved and boiling begins. Observe for the formation of floc in the flask. (Floc is important) Set aside to cool.

4. Add 400 ml water to a flask. Add 50 ml of 10X CMRL 1066 without Glutamine.

5. Add a small stir bar and set on the slowest speed and add the following in order, allow to go into solution before adding the next ingredient:

   1 gm Yeastolate
   3 gm HEPES (store in desiccator)
   2.5 gm Glucose
   0.35 gm Na Citrate
   0.40 gm Na Pyruvate (store in desiccator)
   0.20 gm N-Acetylglucosamine (store in freezer)
   1.1 gm Na Bicarbonate

   Then add the cool Neopeptone solution.

6. Add 25 gm BSA Fraction V. This will not go into solution rapidly, so allow 5-6 hours. DO NOT stir rapidly. It will not help speed the process and it will also damage the value of the BSA. Stir on the slowest speed possible or simply sprinkle the BSA on the top, cover and allow to stand overnight.

7. Adjust pH to 7.6 (critical) with 1N reference buffer.

8. Pre-filter media prior to the .22 μm filtration.

9. Filter sterilize through Millipore .22μm filter. (Sloshing and/or splashing will effect the pH)

10. Add 7 gm gelatin to 100 ml water and dissolve. Cover and autoclave.

11. Add 7% gelatin to the sterile product using aseptic techniques in a hood. The gelatin must be at least room temperature to be fluid.

12. Dispense the media into the proper plastic tubes. Any contaminated tubes will turn yellow after a short incubation period (within 2 days).

Addition of 6% heat inactivated Rabbit serum, 22 μm filtered may be required to supplement satisfactory growth of the spirochetes. Use of serum should be included on an as needed basis and not added to the entire lot of media.

Tubes of media should be inoculated with 20 - 50 μl from a mature culture. Checks for viability can be done at intervals of several days using a dark field microscope. Good growth should be achieved within one week of incubation at 32.5°C.

APPENDIX B
SOLUTIONS

Coomassie Blue Stain (Stock)

C-blue R-250 2 gm
d H2O q.s.200 ml

FILTER

Gel Stain (0.125% C-blue, 50% Methanol, 10% Acetic Acid)

C-blue stock	62.5 ml	31.25 ml
Methanol	250 ml (or)	125 ml
Acetic Acid	50 ml	25 ml
d H2O	q.s.500 ml	q.s.250 ml

FILTER

Destaining solution I (50% methanol;, 10% Acetic acid)

Methanol	500 ml	250 ml
Acetic acid	100 ml (or)	50 ml
d H2O	q.s.1 liter	q.s.500 ml

Destaining solution II (7% acetic acid, 5% Methanol)

Acetic Acid	700 ml (or)	350 ml (or)	140 ml
Methanol	500 ml	250 ml	100 ml
d H2O	q.s.10 liters	5 liters	2 liters

APPENDIX C
FLAGELLA GENE NUCLEOTIDE SEQUENCE OF *BORRELIA BURGDORFERI*.

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        10         20         30         40         50         60
 241 ---------- ------ATGA TTATCAATCA TAATACATCA GCTATTAATG CTTCAAGAAA
 301 TAATGGCATT AACGCTGCTA ATCTTAGTAA AACTCAAGAA AAGCTTTCTA GTGGGTACAG
 361 AATTAATCGA GCTTCTGATG ATGCTGCTGG CATGGGAGTT TCTGGTAAGA TTAATGCTCA
 421 AATAAGAGGT TTGTCACAAG CTTCTAGAAA TACTTCAAAG GCTATTAATT TTATTCAGAC
 481 AACAGAAGGG AATTTAAATG AAGTAGAAAA AGTCTTAGTA AGAATGAAGG AATTGGCAGT
 541 TCAATCAGGT AACGGCACAT ATTCAGATGC AGACAGAGGT TCTATACAAA TTGAAATAGA
 601 GCAACTTACA GACGAAATTA ATAGAATTGC TGATCAAGCT CAATATAACC AAATGCACAT
 661 GTTATCAAAC AAATCTGCTT CTCAAAATGT AAGAACAGCT GAAGAGCTTG GAATGCAGCC
 721 TGCAAAAATT AACACACCAG CATCGCTTTC AGGGTCTCAA GCGTCTTGGA CTTTAAGAGT
 781 TCATGTTGGA GCAAACCAAG  ATGAAGCTAT TGCTGTAAAT ATTTATGCAG CTAATGTTGC
 841 AAATCTTTTC TCTGGTGAGG GAGCTCAAAC TGCTCAGGCT GCACCGGTTC AAGAGGGTGT
 901 TCAACAGGAA GGAGCTCAAC AGCCAGCACC TGCTACAGCA CCTTCTCAAG GCGGAGTTAA
 961 TTCTCCTGTT AATGTTACAA CTACAGTTGA TGCTAATACA TCACTTGCTA AAATTGAAAA
1021 TGCTATTAGA ATGATAAGTG ATCAAAGAGC AAATTTAGGT TCTTTCCAAA ATAGACTTGA
1081 ATCTATAAAG GATAGTACTG AGTATGCAAT TGAAAATCTA AAAGCATCTT ATGCTCAAAT
1141 AAAAGATGCT ACAATGACAG ATGAGGTTGT AGCAGCAACA ACTAATAGTA TTTTAACACA
1201 ATCTGCAATG GCAATGATTG CGCAGGCTAA TCAAGTTCCC CAATATGTTT TGTCATTGCT
1261 TAGATAA---
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