Experimental Infection of Neonatal Calves with *Borrelia burgdorferi*

Karen A. Molinari Clark, MS; Mazhar I. Khan, DVM, PhD; Herbert J. VanKruiningen, DVM, PhD, MD; and Sandra L. Bushmich, MS, DVM

ABSTRACT

Four neonatal calves were inoculated intraperitoneally, intramuscularly, subcutaneously, and intradermally with a total of 2×10^6 organisms from a low passage culture of *Borrelia burgdorferi*. Two control calves were inoculated with sterile BSK media in a similar manner. The six experimental calves were maintained for 7 to 8 weeks. The *B. burgdorferi* infected calves demonstrated an erythematous skin lesion (from which *B. burgdorferi* could be cultured) at the site of inoculation 2 to 3 weeks following infection. Rashes resolved spontaneously within 1 to 2 weeks. Unilateral lymphadenopathy of the left prefemoral lymph node occurred concurrent with the development of skin lesions and persisted throughout the study in all four infected calves.

All infected calves developed a serological response to *B. burgdorferi*, as determined by immune fluorescent antibody (IFA) test and Western blot (WB) analysis. Dissemination of *B. burgdorferi* occurred in all infected calves. *B. burgdorferi* were detected, by culture and/or PCR, in urine, blood, synovial fluid, and tissue samples (kidney, bladder, spleen, and tarsal synovium). Minimal histopathological changes occurred in the infected calves. The most common lesion was a lymphocytic infiltrate noted in skin; scattered lymphocytic foci also occurred in renal cortex, heart, synovial tissue, and liver. This study has demonstrated that neonatal calves experimentally inoculated with *B. burgdorferi* develop disseminated infection, shed *B. burgdorferi* in the urine and produce a specific serological response to the organism.

Key words: *Borrelia burgdorferi*, cattle

INTRODUCTION

Lyme disease is caused by the spirochete *Borrelia burgdorferi* and transmitted by *Ixodes spp* ticks.[1] The disease has been recognized in humans and in a number of domestic animal species including cattle, horses, sheep, dogs, and cats.[2-12] In livestock the pathogenesis and transmission of this disease remain poorly defined. Disease expression among cattle, as well as other domestic animal species, varies. Many animals remain clinically normal while demonstrating antibodies to *B. burgdorferi*.[6,7,13] Clinical signs of Lyme disease reported in cattle include lameness, stiffness, swollen joints, laminitis, arthritis, fever, weight loss, decreased milk production, erythematous skin rash and abortion.[3-9]

Diagnosis of Lyme disease is often difficult and is based on clinical signs, exclusion of other diseases, serological response to *B. burgdorferi* antigens and response to antibiotic therapy.[6-9,13] Positive serology is helpful in making a diagnosis, but it indicates previous exposure only, not necessarily active or clinical infection.[5,6] In cattle it has been suggested that antibody testing may not be as dependable as in other domestic animals due to cross-reacting antibodies to other *Borrelia spp* (*Borrelia coriaceae* and *Borrelia theileri*) or perhaps rumen spirochetal organisms.[5,6] It has been reported that cattle with confirmed infection (ie, *B. burgdorferi* organisms demonstrated) may develop only low antibody titers.[4,5] Definitive diagnosis depends on the demonstration of *B. burgdorferi* in body fluids or tissues of infected animals. This is often difficult due to: sparse numbers of organisms in affected fluids or tissues, difficulty in recovering the organisms because of special media requirements and low replication time, and

From the Department of Pathobiology, The University of Connecticut, Storrs, CT.

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Address reprint requests to Sandra L. Bushmich, MS, DVM, Department of Pathobiology, U-89, The University of Connecticut, 61 North Eagleville Road, Storrs, CT 06269-3089.

the need for darkfield microscopy or special stains to demonstrate the organisms.[6,7,9,13-15]

The objective of this study was to further characterize bovine serological and pathological responses to *B. burgdorferi*, as well as determine patterns of organism dissemination in tissues and body fluids by utilizing an experimental calf model.

MATERIALS AND METHODS

Animals

Neonatal Holstein bull calves, weighing approximately 40 kg, were obtained from the University of Connecticut dairy herd, Storrs. Each calf was removed from the dam at birth and not allowed to nurse. Serologic testing was not conducted on the dams. The calves were given oral bovine rotavirus and coronavirus vaccine ("Calf-Guard", Norden Laboratories) and one oral dose of synthetic colostrum diluted to one liter. Synthetic colostrum (Colostrix (454 g), Fisons Animal Health, Minneapolis, MN or First Milk Formula (272 g), Land O' Lakes, Ft. Dodge, IA) was tested by Western blot analysis to insure the absence of antibodies to *B. burgdorferi*. Calves were transported to an isolation facility, where they were individually housed in wire pens on wood shavings. They were fed milk replacer (Blue Seal Lawrence, MA) for 2 weeks, then grain and hay were added to their diet *ad libitum*.

Inoculum

A second or third passage Connecticut *B. burgdorferi* tick isolate (Provided by Dr. John Anderson, Connecticut Agricultural Experimental Station, New Haven, CT) was used for animal inoculations. Experimental injections contained approximately 2×10^6 log phase organisms suspended in Barbour-Stoenner-Kelly (BSK) medium. Cell counts were determined using a bacterial counting chamber (Petroff Hausser bacterial counting chamber, Hausser Scientific, Blue Bell, PA).

Experimental plan

Prior to inoculation all calves were determined to be free of *B. burgdorferi* antibodies (IgG) by immune fluorscent antibody (IFA) and Western blot analyses; urine culture for *B. burgdorferi* was negative. Four calves (calves 1-4) were inoculated with *B. burgdorferi* at 6 days of age in the left flank as follows: following surgical skin preparation (hair clipped, site scrubbed with tamed iodine followed by alcohol wipes 3 times, then allowed to dry prior to procedure), 0.5 mL of the inoculum was administered intraperitoneally, intramuscularly, subcutaneously, and intradermally for a total inoculum volume of 2 mL. Two control calves (calves 5 and 6) were inoculated in a similar manner with an equal volume of sterile BSK medium.

Daily physical examination of all calves included: rectal temperature, pulse and respiration rate, evaluation of attitude, appetite and gait, visual examination of feces, palpation of peripheral lymph nodes, examination of joints for swelling, and of skin for erythematous skin lesions. Circumferences of both carpi and tarsi were measured on a weekly basis. Blood was collected 3 times weekly aseptically from the jugular vein. Blood analyses included packed cell volume (PCV), total protein (TP), dark field examination and culture (3 times per week), PCR for *B. burgdorferi* (once a week) and chemistry screen (weeks 0, 3, and 7). Midstream urine was collected 3 times a week and analyzed by darkfield microscopy, culture, and fluorescent antibody (FA) testing; weekly samples were analyzed by PCR/slot blot for *B. burgdorferi*. Four millimeter skin biopsies of dermis and subcutis were taken from aseptically prepared erythematous skin lesions of infected calves 3 and 4. A carpal synovial fluid sample was taken at the time of the skin biopsy. Control calves 5 and 6 received similar skin biopsy and arthrocentesis 2 weeks post-inoculation. Calves were maintained for 40-60 days then killed by IV injection of euthanasia solution (Beuthanasia D, Schering-Plough Animal Health, Kenilworth, NJ). Necropsy and histopathological examination with culture and PCR of selected tissues was done on each calf.

Serology

Weekly serological responses to *B. burgdorferi* were evaluated by immune fluorescent antibody test and Western blot analysis.

Immune fluorescent antibody

A previously described IFA technique was used.[16] Multi-well acetone resistant slides were coated with a single layer of whole *B. burgdorferi* suspension. *B. burgdorferi* used were a cocktail of the isolate used for inoculation plus several other low passage isolates. Calf sera were prepared in two-fold serial dilutions (beginning with 1:32 and ending with 1:1024) and incubated in wells for 30 minutes at 37°C. Slides were rinsed twice in phosphate buffered saline (PBS) and once in distilled water. Fluorescein isothyiocynate (FITC) labeled goat anti-bovine heavy and light chain IgG (Kirkegaard and Perry, Gaithersburg, MD) (diluted 1:400), was applied to each well, incubated and rinsed as before. Slides were examined at 400× using a microscope with a UV light source. A titer of $\geq$ 1:64 was considered positive.

Western Blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed on cultured *B. burgdorferi* using a modification of the method described by Laemeli.[17] Spirochetes were prepared by washing 150 mL of whole cells from log phase cultures of *B. burgdorferi*. three times by centrifugation at 10,000×*g* for 15 minutes in a 0.1% solution of merthiolate and phosphate buffered saline (PBS). Protein concentration was estimated by the Bradford method.[18] Approximately 300 µg of *B. burgdorferi* protein was mixed with 187 of µL sample buffer (0.25 mol/L, 40% glycerol, 2% sodium dodecycl SDS, 20% 2- mercaptoethanol, 0.025% bromophenol blue) and denatured by boiling for 5 minutes. *B. burgdorferi* protein and molecular weight standards (prepared according to manufacturer) (Beuthanasia D, Schering-Plough Animal Health) were subjected to electrophoresis using 10% resolving gels and 4% stacking gels. Electrophoresis was performed at a constant current of 100 mA until dye-front reached 1 cm from the bottom of the gel. Proteins were pre-equilibrated in transfer buffer (25 mM Tris base, 38 mM Glycine, 20% methanol, pH 8.3) for 30 minutes, then transferred onto 0.45 µm nitrocellulose membranes (Bio-Rad, Richmond, CA) at 70 volts for 2.5 hours using a Trans-blot Electrophoretic Transfer Cell (Bio-Rad) as previously described by Towbin *et al.*[19] Following transfer, nitrocellulose membranes were stained in Ponceau's stain (Sigma Chemical Co., St. Louis, MO), for approximately 10 minutes to determine if proteins were properly transferred. The membranes were cut into strips, placed in a small tray and blocked with 2% BSA and 1% horse serum in Tris buffered saline for one hour at room temperature with rocking. Strips were rinsed (three 10 minute washes) in wash buffer (0.85% NaCl, .12% Tris, .05% Tween 20), overlaid with experimental calf sera (diluted 1:100) and incubated as before for two hours. Serum was removed, strips were washed as described and goat anti-bovine heavy and light chain IgG phosphatase labeled conjugate (Kirkegaard and Perry, Gaithersburg, MD) (diluted 1:2500) was added and incubated for one hour. The strips were rinsed as before and reacted with BCIP/NBT phosphatase substrate (Kirkegaard and Perry) until optimal protein band development was achieved (1-10 minutes). The reaction was stopped by the removal of substrate and rinsing the strips several times with distilled water.

ANTIGEN TESTING

Culture

All urine, blood, synovial fluid, skin biopsies and postmortem tissues and fluids (kidney, bladder, testis, spleen, heart (myocardium and septum), brain (cerebrum, cerebellum), prefemoral lymph nodes, synovial tissue (carpi and tarsi), pericardial fluid, cardiac blood, urine, aqueous humor, synovial fluid, and CSF were cultured for *B. burgdorferi* in Barbour-Stoenner-Kelly medium as previously described.[14] Tubes were inoculated in tandem, using one tube of media without antibiotics and one with ciprofloxacin (40 mg/mL) and rifampicin (20 mg/mL) added to retard the growth of contaminants. Approximately 0.5 mL of fluid or 15-20 mg of minced tissue sample was inoculated into 7 mL of sterile BSK media. Samples were incubated at 32°C and examined by darkfield microscopy after one week incubation, then biweekly for one month. Identity of spirochete positive cultures was confirmed by PCR analysis.

Darkfield examination

Darkfield microscopy was used to examine blood, urine, and synovial fluid. One drop of fluid was examined at 200× and 400× magnification. Before examining fresh urine, approximately 20-50 mL was centrifuged at 11,000×*g* for 20 minutes, all but 3 mL of the supernatant was poured off and discarded. The remaining fluid was mixed with the sediment then examined microscopically.

Fluorescent antibody staining

Direct fluorescent antibody staining was used to test all fluids excluding premortem blood samples. Two separate drops of fluid were placed on a glass microscope slide, air dried, then fixed in acetone for 10 minutes. Urine was prepared as described above before testing. Approximately 2 drops of fluorescein conjugated polyclonal goat antiBorrelia antibody (Kirkegaard and Perry) (1:20 dilution) was applied to each droplet. Slides were incubated at room temperature in a moisture chamber for 30 minutes, then placed in PBS for 10 minutes and rinsed in distilled water three times. Slides were examined at 200× and 400× using a microscope with a UV light source for the presence of stained spirochetes.

Polymerase chain reaction

Polymerase chain reaction (PCR) was used to detect *B. burgdorferi* in weekly urine and blood samples as well as the following postmortem samples: kidney, bladder, spleen, prefemoral lymph nodes, heart (septum and myocardium), cerebrum, synovial fluid, and CSF. Chromosomal DNA was extracted from body fluids and tissues as previously described.[20] The primer pair used amplifies a specific 309 base pair segment of *B. burgdorferi* outer surface protein A (OspA) and have been previously described by Malloy et al.[21] This primer set will not amplify Osp A of other *Borrelia spp*, including *B. coreaceae* a pathogen of cattle. Primers were synthesized by the Biotechnical Services at the University of Connecticut, Storrs. A GeneAmp PCR reagent kit (Perkin-Elmer Cetus, Norwalk, CT) was used to amplify gene products as previously described.[20] Triplicate posi-

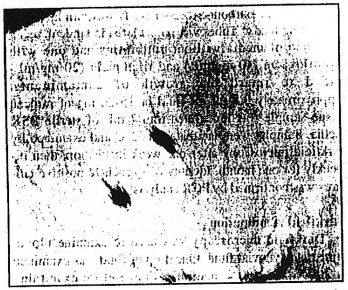


Fig 1: Erythematous skin rash in a B. burgdorferi *infected calf at the site of inoculation; sutures indicate biopsy sites.*

tive and negative controls were included in each PCR run; PCR sample preparation and conduction were conducted in a laboratory dedicated to this purpose.

Slot blot hybridization

PCR amplified DNA product of *B. burgdorferi* (200 ng) was used as DNA probe for slot blot hybridization. *B. burgdorferi* DNA was digested using 10 units of EcoRI in 20 µL of reaction buffer.[22] The digested DNA was heat denatured (95°C for 5 minutes) and end labeled with [gamma-32P]-dATPI using a Gibco BRL 5' DNA terminus (Gibco BRL, Life Technologies, Gaithersburg, MD).

Amplified DNA products (1 µg) were digested using EcoRI in reaction buffer. Samples were incubated with the enzyme for one hour at 37°C, followed by the addition of 6 X SSC (0.9 M NaCl, 0.09 M sodium citrate pH 7.0) was added and the digested DNA was heat denatured at 95°C, 5 minutes. The samples were applied to a Zeta Probe membrane (Bio-Rad)) using a slot blot manifold (Bethesda Labs, Bethesda, MD) under vacuum pressure. The membrane was air-dried, baked for 2 hours at 80°C and sealed inside a plastic bag with prehybridization buffer (10× denhardts solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, and 0.2% BSA), 6× SSC, 20 mM sodium phosphate, 4% SDS, salmon sperm DNA (80 µg/mL)) and incubated for 60 minutes at 59°C.

The labeled DNA probe, heat denatured at 95°C for 5 minutes, was added with fresh prehybridization buffer to the Zeta-Probe membranes and allowed to hybridize on a rocker overnight at 59°C. The membranes were removed from the bag and washed once in a solution made up of 3× SSC, 1% SDS and distilled water at room temperature for 30 minutes. This was followed by a second wash in a solution made up 1× SSC, 1% SDS and distilled water at room temperature for 30 minutes. The membranes were washed for a final time in a solution made up of 0.1× SSC, 1% SDS and distilled water at 60°C for 45 minutes. Membranes were air dried and exposed to x-ray film for approximately 24 hours using an intensifying screen at -70°C. [22]

Pathology/histopathology

Necropsy was conducted immediately following euthanasia. Selected tissues (listed previously) were collected aseptically for *B. burgdorferi* culture. Tissues from all major organ systems, excluding endocrine, were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 micrometers and stained with H & E. Selected tissues with lesions were stained with the Modified Steiner method[23] and by immunocytochemistry (ICC)[24] (Ying Liu, MS thesis, Storrs, CT) for the presence of spirochetes. In the latter, polyclonal rabbit anti *B. burgdorferi* serum (ViroStat, [code No. 0301], Portland, ME) at a dilution of 1:20,000 was applied, followed by the addition of the secondary antibody, biotinylated goat anti-rabbit (dilution 1:500), and then a streptavidin-horseradish peroxidase conjugate. Amino-ethylcarbazole (AEC) served as the chromogen. The tissue sections were then counterstained with Lerner-2 hematoxylin, coated with crystal mount, and the slides were coverslipped.

Statistical analysis

The paired *t* test was used to compare the prefemoral lymph node sizes of the infected and control groups.

RESULTS

Clinical and laboratory findings

Calves remained bright and alert with good appetites throughout the study. Rectal temperature, pulse and respiration rates remained within normal ranges in both infected and control group calves. Three of the four infected calves (1, 3 & 4) and both control calves (5 & 6) had mild diarrhea, which developed between weeks 1 and 3 of infection and resolved following oral electrolyte treatment. Other clinical disorders which occurred in the calves include: a cough from week 1-7 in calf 1, an episode of mild bloat in calf 3 (week 7) which resolved following stomach intubation, and a soft tissue abscess in the buccal mucosa of calf 4 (days 23-41), which drained and healed spontaneously; abscess material was not cultured.

Erythematous skin lesions appeared at the site of inoculation (left flank) of all four *B. burgdorferi* infected calves 2 to 3 weeks following infection. The skin lesions first appeared as small red macules, which expanded to 5 to 10 cm in diameter (Fig 1). Lesions faded within 1 to 2 weeks. Lymphadenopathy of the left prefemoral lymph node (near the inoculation site) became apparent near the onset of skin lesion development and remained until necropsy in all *B. burgdorferi* infected calves. Control calves did not develop skin rash or lymphadenopathy at the site of inoculation. There was no difference in carpal and tarsal circumference between *B. burgdorferi* infected and control groups. Lameness was not observed in any of the calves.

Table 1
Immune fluorescent antibody (IFA) test and Western blot (WB) values for B. burgdorferi *infected calves (calves 1-4) and noninfected control calves (calves 5 & 6). IFA results are shown as inverse titers, 1:64 is considered a low positive. WB results are shown as antibodies to 41, 39, 34 and 31 kDa proteins of* B. burgdorferi.

Week Following Inoculation	0	1	2	3	4	5	6	7
Calf 1								
IFA	-	-	-	32	64	64	128	128
WB	41	41	41	41,34,31	41,39,34,31	41,39,34,31	41,39,34,31	41,39,34,31
Calf 2								
IFA	-	-	-	32	64	64	128	128
WB	41	41	41	41	41,34	41,39,34,31	41,39,34,31	41,39,34,31
Calf 3								
IFA	-	-	-	32	64	64	128	256
WB	-	-	-	-	41,34,31	41,39,34,31	41,39,34,31	41,39,34,31
Calf 4								
IFA	-	-	-	32	64	64	64	64
WB	41	41	41	41	41	41,34	41,39,34	41,39,34,31
Calf 5								
IFA	-	-	-	32	32	32	32	32
WB	41,39	41,39	41,39	41,39	41,39	41,39	41,39	41,39
Calf 6								
IFA	-	-	-	32	32	32	32	64
WB	41,39	41,39	41,39	41,39	41,39	41,39	41,39	41,39

Serum biochemical values were within normal limits, with the following two exceptions. Serum globulin levels were depressed in all calves (the average value of all calves was 1.4 vs the normal range of 3.0 to 3.5 g/dL) and alkaline phosphatase was moderately elevated in all calves. In both infected and control calves packed cell volumes remained within normal range throughout the study (average value of all calves was 35.4% vs the normal range of 24% to 48%, Schalm's Veterinary Hematology[26]) but total protein levels were slightly below normal reference range (average value for calves was 4.8 vs the normal reference range of 5.6 to 6.8 g/dL, Schalm's Veterinary Hematology [26]).

Serology

Table 1 summarizes the antibody response to *B. burgdorferi* in all calves. All calves were seronegative by IFA and Western blot analysis prior to experimental inoculation. The four infected calves developed a positive IFA antibody titer (≥ 1:64) by the fourth week of infection, which increased throughout the study in calves 1 thru 3. Calf 4 maintained a 1:64 titer from weeks 4 to 7. Control calf 5 remained IFA negative (≤ 1:64) throughout the trial. Control calf 6 developed a positive antibody titer (1:64) 7 weeks following experimental inoculation; however Western blot analysis of its sera did not reveal antibodies specific to *B. burgdorferi*.

Western blot analysis of sera from all 4 infected calves demonstrated a sequential development of antibodies to *B. burgdorferi* specific outer surface proteins A and B (OspA, 31 kDa and OspB, 34 kDa) (Table 1 and Fig 2A). In calves 1 thru 3 these antibodies were detectable 3 to 4 weeks following experimental infection. The serological response to these proteins was delayed and weak in calf 4. In this calf antibody to the 34 kDa protein was not detectable until the fifth week postinoculation and an antibody response to the 31 kDa protein was detectable at week seven postinoculation (Table 1). Antibodies directed against the nonspecific 41 kDa flagellar protein and the reportedly *B. burgdorferi* specific 39 kDa protein were apparent in both infected and control calves (Table 1 and Figs 2A and 2B). Antibody response to the flagellar (41

Table 2.
Urine culture and PCR results for B. burgdorferi *infected calves (calves 1-4) and noninfected control calves (calves 5 & 6).*

Week of Inoculation	0	1	2	3	4	5	6	7
Calf 1	-	+P	+P	+P	-	-	-	-
Calf 2	-	+P	+P	-	+P	-	+C,P,FA	+C,P
Calf 3	NS	-	-	-(*) (**)	+P	+P	-	-
Calf 4	-	-	-	-(*)	-	+P (***)	+P (***)	+P
Calf 5 (control)	-	-	-	-	-	-	-	-
Calf 6 (control)	-	-	-	-	-	-	-	-

NS=No Sample (There was no sample available for PCR/slot blot; the sample was examined by FA and darkfield microscopy and was negative for Borrelia burgdorferi*).*
*C= culture; P= PCR; FA= Fluorescent antibody staining; *Skin biopsy (+) for Bb (culture and PCR); **Synovial fluid sample for PCR (+); ***Blood sample PCR (+); Note: Identity of all spirochete positive cultures was confirmed by PCR/slot blot hybridization*

kDa) protein remained weak throughout the course of infection in all calves. Response to the 39 kDa protein became stronger with time in all of the infected calves, but remained weak in the control calves (Figs 2A and 2B). Immunoblots of the synthetic colostrum fed to all calves revealed antibody to the nonspecific 41 kDa flagellar protein only.

Antigen testing

B. burgdorferi was detected in urine of all 4 experimentally infected calves. Results of urine culture and PCR (demonstrated by slot blot hybridization) are presented in Table 2. *B. burgdorferi* was also detected by PCR in occasional blood and synovial fluid samples taken from infected calves, and by culture and PCR in all skin biopsy samples taken from infected calves (Table 2). All control calf samples were PCR and culture negative. Direct darkfield microscopy of urine, blood, or synovial fluid samples did not reveal spirochetes in either infected or control calves. Direct fluorescent antibody (FA) staining revealed spirochetes in the urine of calf 2 during week 6 of infection; FA staining of all other samples was negative (Table 2).

At necropsy, *B. burgdorferi* was isolated by culture from kidney and bladder of calf 2, and spleen and left hock synovial tissue of calf 4. All positive cultures were confirmed as *B. burgdorferi* by PCR/slot blot hybridization. PCR performed directly on tissues and body fluids collected post-mortem (listed previously) did not detect *B. burgdorferi*.

Necropsy and histopathologic results

At necropsy the sites of inoculation were well healed and without erythema or thickening. In all 4 *B. burgdorferi* infected calves the left (side of inoculation) prefemoral lymph node was significantly ($p \leq 0.01$) larger than the right prefemoral lymph node (Fig 3). One of the four *B. burgdorferi* inoculated calves had a buccal ulcer that extended from the mucosa of the left cheek through to the epidermis, consisting primarily of a granulocytic infiltrate. Histologically, the 4 calves inoculated with *B. burgdorferi* had lymphocytic, plasmacytic infiltration at the site of inoculation in the left flank; control calves did not. This appeared predominately in-between hair follicles and around blood vessels. The enlarged left prefemoral lymph node of one infected calf had cortical hyperplasia and that of another infected animal had sinus histocytosis. The other 2 infected calves' prefemoral lymph nodes were no different histologically than those of controls.

Three of the 4 infected calves had focal tonsilitis, characterized by granulocytes and foreign material in a few crypts; so did both controls. Three of the four infected subjects had focal patchy interstitial pneumonitis, a change not seen in the two controls. One infected calf had few foci of lymphocytic nephritis bilaterally; one had a few foci of lymphocytic synovitis in one hock joint; and another had few focal lymphocytic aggregates in portal triads of the liver and subendocardially in the interventricular septum of the heart.

These changes were not observed in the control calves. Spirochetes were not demonstrable in the discussed foci when stained by Steinner's silver method or immunocytochemistry.

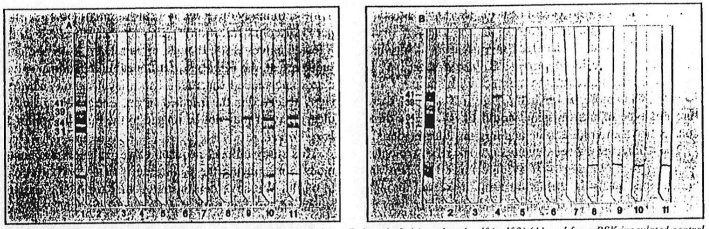
DISCUSSION

In this study we have utilized an experimental calf model to further define the pathogenesis of *B. burgdorferi* infection in cattle. Neonatal calves were susceptible to infection as demonstrated by the development of an erythematous rash at the site of *B. burgdorferi* inoculation, a serological response to *B. burgdorferi* and by detection of *B. burgdorferi* in body fluids and tissues.

The development of an erythematous rash at the site of inoculation was the only significant clinical sign observed and was consistent in all infected calves. In humans, erythema chronicum migrans (ECM) is a hallmark of *B. burgdorferi* infection and ECM dermal tissue is a good source for recovering *B. burgdorferi*, as was also shown in this study.[26,27] Erythematous skin lesions have been reported in naturally infected cattle.[8] They are rarely seen during spontaneous infection of other animal species, possibly because of hair covering; however spirochetes have been isolated from the skin of infected animals lacking a rash.[28,29] Lymphocytes, the major cellular component of human ECM,[30] were observed as the main cellular infiltrate at the site of *B. burgdorferi* inoculation in all four infected calves.

Use of a tick mode of infection, as well as a greater number of experimental animals, may have produced additional clinical signs. Distinct differences in clinical and serological response have been observed in dogs experimentally inoculated with cultured *B. burgdorferi* compared to those exposed experimentally via *B. burgdorferi* infected ticks.[29]

Disseminated *B. burgdorferi* infection occurred in all infected calves. *B. burgdorferi* was demonstrated in the urine, blood, premortem synovial fluid, and in several tissues taken postmortem (blood, kidney, bladder, spleen, and synovial tissue). Pathological changes observed in skin, lung, lymph node, heart, liver, and synovium of the infected calves may also represent a response to disseminated *B. burgdorferi* infection; control calves did not have similar lesions at these sites. In this study, *B. burgdorferi*.



Figs 2A and 2B: Western Blot analysis of antibody to B. burgdorferi *in a* B. burgdorferi *inoculated calf (calf 2) (A) and for a BSK inoculated control calf (calf 6) (B). Serum was tested weekly from day 0 to post-inoculation week 7. The results in each immunoblot lane are as follows; lane 1: positive control experimentally* B. burgdorferi *infected calf serum; lane 2: negative control noninfected calf serum; lane 3: conjugate control goat anti-bovine H&L chain IgG (diluted 1:2500); lane 4: pre-inoculation; lane 5: week 1; lane 6: week 2; lane 7: week 3; lane 8: week 4; lane 9: week 5; lane 10: week 6; lane 11: week 7. Molecular weights are in kilodaltons (kDa).*

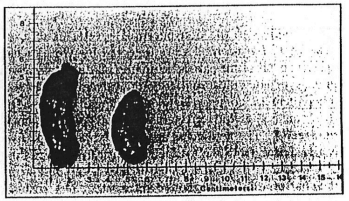


Fig 3: Comparison of left (on left) and right (on right) prefemoral lymph nodes from a B. burgdorferi *inoculated calf (calf 2) at necropsy.*

was more readily detected in pre-mortem urine and blood samples by PCR than by culture or FA staining; however, culture was more successful than direct PCR on tissue samples collected at necropsy. Factors involved may include the concentration of urine by high speed centrifugation prior to PCR, the sparse numbers of organisms in tissues, and the ability of PCR to detect nonviable organisms.[31]

Spirochetes were detected in the urine of all infected calves but in the blood of only one. Early or intermittent spirochetemia may have contributed to the difficulty in demonstrating *B. burgdorferi* in the blood of the calves.[32,33] The pattern of urine shedding of *B. burgdorferi* in infected calves was variable and persisted over a period of weeks in some calves. Positive urine cultures obtained demonstrate that live organisms are present in the urine of infected calves. More frequent analysis of urine and blood

may provide a clearer pattern of urine shedding and spirochetemia. The demonstration of *B. burgdorferi* in the urine of infected cattle has lead investigators to speculate that perhaps, like leptospirosis, *B. burgdorferi* may be transmitted among cattle by contact with urine.[4,7,9,34] *B. burgdorferi* is more labile than *Leptospira spp* and, therefore this type of transmission would likely require direct contact between voided urine and mucous membranes.[6]

The 4 infected calves developed a specific serological response to *B. burgdorferi* as determined by IFA and western blot analysis. Antibody titers increased throughout the course of infection in all infected calves with the exception of calf 4. Calf 4 produced a low antibody titer and its response to Osp A and B was both weak and delayed compared to the other infected calves. This calf also demonstrated the most widely disseminated infection; *B. burgdorferi*. was detected in its blood, antemortem urine, and several tissues post-mortem. The phenomenon of *B. burgdorferi*. infection with weak serological response has also been observed in natural disease.[4,5,35]

Antibodies to the nonspecific flagellar protein (41 kDa) of *B. burgdorferi* were detected by Western blot analysis in all of the calves beginning on day 0, as well as in the synthetic colostrum fed to the calves. Antibodies to this protein are common in adult cattle and probably represent antibodies formed against other flagellated flora in ruminants.[5,6] The antibodies appear to have been passively transferred from the synthetic colostrum. The positive IFA titer seen in control calf 6 is also a response to the flagellar protein, as supported by western blot analysis. An antibody response to the reportedly *B. burgdorferi* specific 39 kDa protein[36] was also common to both *B. burgdorferi* infected calves and noninfected control calves. This response in the infected calves became more distinct throughout the course of infection but remained weak in the control calves. Antibody to the 39 kDa has been commonly found in sera of cattle from nonendemic regions and therefore, may not be a specific marker of *B. burgdorferi* infection in cattle (Bushmich, unpublished data). In human disease, the presence of antibodies to the 39 kDa protein is commonly seen in patients with late manifestations of disease but antibodies against this protein have also been found on occasion, in patients with syphilis suggesting that there may be cross-reactive epitopes within this protein.[37]

The serum chemistry panels in both infected and control calves did not reveal any major organ dysfunction, and hematocrit levels were within normal range. Depressed serum globulin and total protein levels may reflect lower levels of immunoglobulins present in synthetic compared to natural colostrum. Alkaline phosphatase elevations may be due to elevated bone isoenzyme commonly observed in young animals (W Hoffman, personal communication).[38] The diarrhea observed in the infected calves is not thought to be related to, or the consequence of, the *B. burgdorferi* inoculation; control calves also experienced diarrhea. The miscellaneous clinical signs observed in the infected calves (cough, bloat, buccal ulcer) may or may not be related to the introduction of *B. burgdorferi*.

This study utilized neonatal calves as an experimental model for studying the pathogenesis of Lyme borreliosis in cattle. We have demonstrated that following experimental inoculation with *B. burgdorferi* there is a sequential development of antibodies to specific *B. burgdorferi* proteins in these calves. Experimentally infected calves developed an erythematous rash from which *B. burgdorferi* was cultured. They also shed *B. burgdorferi* in urine for several weeks duration and *B. burgdorferi* was disseminated to other organs/tissues. It was demonstrated that calves can be spirochetemic and spirocheturic without the development of clinical disease. Further investigations are needed to determine the pattern and duration of spirochetemia and spirocheturia in cattle as well as clarify the clinical and pathophysiological manifestations associated with *B. burgdorferi* infection in naturally infected cattle.

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