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Intrauterine transmission of *Borrelia burgdorferi* in dogs

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Summary

To determine whether intrauterine transmission of *Borrelia burgdorferi* could exist in dogs, 10 female Beagles were inoculated intradermally with approximately 1,000 *B burgdorferi* on day 1 of proestrus; inoculation was repeated every 2 weeks during the gestation period. Ten female control Beagles were similarly inoculated with phosphate-buffered saline solution. Prior to the start of the study, all females and 3 males used for breeding were seronegative for *B burgdorferi* on the basis of results of the indirect fluorescent antibody test and immunoblot (western blot) analysis. Similarly, results of culture of blood for *B burgdorferi* were negative. All 20 of the females were bred naturally. Blood samples were collected weekly for serologic testing and culture. Blood samples were obtained from live pups on day 1 of life, then weekly until pups were 6 weeks old when they were euthanatized. Tissues were obtained for culture and testing by use of polymerase chain reaction (PCR). Of 10 spirochete-inoculated (SI) females, 8 became infected with *B burgdorferi* as evidenced by spirochete culture results and/or PCR-detected *B burgdorferi* DNA in the tissues of females or their pups. Of the 10 SI females, 8 delivered litters (3 to 7 pups) that had at least 1 neonatal or 6-week-old pup with *B burgdorferi* DNA-positive tissues (by PCR), and spirochetes were cultured from tissues from pups of 2 litters. Four pups of 3 separate litters (a stillborn, a neonate that survived to 30 minutes of age, a 20-hour-old, and a 48-hour-old) had *B burgdorferi*-positive tissues (by PCR), and the 20-hour-old pup was also culture-positive, indicating intrauterine infection. Further evidence of intrauterine exposure was the presence of IgM antibodies to *B burgdorferi* detectable by western blot in 3 of 7 one-day-old pups that did not receive colostrum, indicating a primary immune response. Eight of 10 SI females and 10 of 10 control females carried litters to term. Differences between SI and control Beagles were seen in the duration of gestation, number of resorptions, and number of dystocias. All control females and pups remained seronegative, culture-negative, and *B burgdorferi*-negative throughout the study.

Intrauterine infection by *B burgdorferi* does occur in dogs and is a potential means by which the spirochete can be transmitted in a breeding population in the absence of a tick vector.

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Lyme disease is a multisystemic syndrome in human beings caused by the spirochete *Borrelia burgdorferi*.[1,2] In northeastern and northwestern United States, the spirochete is transmitted by the tick, *Ixodes dammini*.[3-5] Lyme disease has been rapidly spreading from its initial endemic foci of infection in northeastern United States and Wisconsin, and has now been reported in 48 states.[6] *Borrelia burgdorferi* infection has been reported in dogs, and some develop clinical signs of disease, such as fever, inappetence, lethargy, acute onset of lameness, generalized signs of pain, and lymphadenopathy.[7] If left untreated, dogs may develop arthritis, renal involvement, and myocarditis.[7-9]

Maternal-fetal transmission of *B burgdorferi* has been reported in horses, human beings, coyotes, and *Peromyscus leucopus*.[10-13] In human beings, maternal infection during pregnancy has been associated with fetal death, congenital cardiac defects, early neonatal death, cortical blindness, syndactyly, and a neonate delivered with erythema chronicum migrans.[11,14-16] Detection of spirochetes from a coyote fetus indicated that intrauterine infection may exist in canids.[12]

To the authors' knowledge, the effects of *B burgdorferi*, if any, on pregnancy in dogs are not known. Study of intrauterine transmission of *B burgdorferi* is important because of the potential for practical, biologic, and epizootiologic implications. Intrauterine infection of fetuses may be a means of transmitting *B burgdorferi* in the absence of a tick vector. The objectives of the study reported here were to determine whether intrauterine infection was possible, whether *B burgdorferi* infection has an effect on

pregnancy in bitches, and to determine the effects of intrauterine infection on fetuses and neonates.

Materials and Methods

Dogs—Three adult male and 20 adult female Beagles were obtained from a commercial source.[a] All dogs were related, because they were derived from an original Beagle colony. All dogs were free of arthropods on arrival, and were seronegative for *B burgdorferi* on the basis of results of the indirect fluorescent antibody (IFA) test at serum dilution of 1:32 (5 log 2) and by immunoblot (western blot [WB]) analysis. The males were proven breeders (sired previous litters and had a recent semen quality evaluation with at least 80% motility, 70% morphologically normal spermatozoa, and 70 million spermatozoa/ml). All females had a history of having at least 1 clinically normal litter. All dogs were housed at the University of Wisconsin-Madison School of Veterinary Medicine animal care facility. Females were housed in individual rooms (11 × 11 ft) with resting platforms. Approximately 1 week prior to parturition, a whelping box (41 × 25 × 7 in) was placed in each room. Males were housed in individual runs (61.5 × 86 in).

Study design—Females (n = 20) were entered into the study on the first day that proestrus was detected by results of vaginal cytologic examination.[17] The 3 males were bred to the control and spirochete-inoculated (SI) females. The control females were bred first to limit the possibility of exposure to *B burgdorferi*. Control females were bred in late winter to spring (Jan 14 to Mar 14), and the SI females were bred in late summer to fall (July 4 to October 1).

Ten female controls were inoculated (on the first day of proestrus) intradermally with 0.5 ml of phosphate-buffered saline solution (PBSS) minus the spirochetes and 10 more were inoculated intradermally with approximately 1,000 spirochetes, suspended in 0.5 ml of PBSS, on a shaved skin patch on the dorsum of the neck. The inoculation procedure was repeated on each female every 2 weeks until parturition. Blood samples were collected at 7-day intervals, beginning on day 1 of proestrus. Blood was taken from the cephalic vein of each female for serologic testing and culture until 6 weeks after parturition. Females were evaluated on the progress of the estrous cycle every other day, using vaginal cytologic findings as an indicator. The first day of estrus was defined as the first day that a vaginal smear contained > 70% superficial epithelial cells. On days 1 and 3 of estrus, each female was allowed to mate naturally with 1 of the males. All 3 males were used to breed the control and SI females equally on a regularly alternating basis. On days 21, 28, 35, and 42 after the first mating, each female was examined for pregnancy, using abdominal palpation and ultrasonography; the number of fetuses was estimated at that time. The presence of a living fetus was determined by observation of amniotic vesicles containing a beating heart. Pups were observed for 6 weeks after parturition. Newborn pups were examined twice daily to be sure they were nursing. Room temperature was adjusted to 80 F during the first 2 postpartum weeks. Any pups that were not nursing were fed by way of an orogastric tube every 4 hours and kept under a heat lamp at 85 to 90 F. Dehydration was corrected by SC administration of warmed (95 F) lactated Ringer's solution. There was limited success in saving pups that were weak within the first 24 hours. Blood samples were collected from the jugular vein within the first 24 hours, then on days 8, 15, 22, 29, and 42. Blood was obtained from most pups after they had nursed (and therefore received colostrum) to minimize the stress of sample collection and handling. These samples were cultured and tested for antibodies to *B burgdorferi* by use of WB and IFA. After blood sample collection on the 42nd day, pups were euthanatized by use of a euthanasia solution[b] and were necropsied. All stillborn pups or those that died before the study was complete were necropsied. The skin was surface-disinfected with 95% alcohol, then was reflected. Tissues collected by use of sterile instruments from all pups were liver, spleen, lymph nodes, brain, heart, kidneys, lungs, thymus, urinary bladder, and CSF. Tissues were fixed in buffered 10% formalin and were embedded in paraffin. Sections, 7 μm thick were cut, followed by staining with H&E. Freshly collected tissues also were tested by polymerase chain reaction (PCR) and culture. The females were euthanatized by use of the aforementioned euthanasia solution and were necropsied at 6 weeks after parturition. Tissues collected from females were ovaries, uterus, cervix, liver, spleen, lymph nodes, brain, heart, kidneys, bladder, mammary glands, and CSF. Uterine implantation sites were enumerated in the endometrium of the uterus in each female. Tissues were saved for culture, histologic examination, and PCR as described for the pups.

Spirochete—The spirochete used for inoculation was the second passage of a *B burgdorferi* isolate obtained from an adult *I dammini*.[c] After growth in BSK II medium at 33 C,[18] the spirochetes were enumerated, using an improved Neubauer hemocytometer.[d] Concentrated spirochetes were counted (approx 10^6 cells/ml), then were diluted to the appropriate amount. Diluted cells were then recounted and adjusted to approximately 1,000 spirochetes/0.5 ml of PBSS.

Spirochete isolation—Isolation attempts for *B burgdorferi* were performed by inoculating culture tubes containing 4.0 ml of BSK II medium with 0.1 ml of blood and incubating at 33 C. Tissue specimens were minced with scissors and placed in 4 ml of BSK II medium with antibiotics (25 μg of rifampin and 10 μg of phosphomycin/ml).[e] Samples were examined for spirochetes weekly for 12 weeks by use of

[a] Ridgland, Mt Horeb, Wis.

[b] Beuthanasia D, Schering Corp, Kenilworth, NJ.
[c] Supplied by Dr. E. Bosler, New York State Department of Health, Stony Brook, NY.
[d] Reichert Scientific Instruments, Buffalo, NY.
[e] Sigma Chemical Co, St Louis, Mo.

dark-field microscopy. Any spirochetes were identified by use of the PCR.

Polymerase chain reaction—To determine the sensitivity of the PCR, BSK II media was seeded with 10^7 organisms of the New York tick isolate of *B burgdorferi*/ml. Serial dilutions (tenfold) were made to final density of 1 organism/ml. To determine the specificity of the PCR, several isolates of *B burgdorferi*, as well as other *Borrelia* spp were tested for amplification. The *B burgdorferi* isolates tested included 3 from *I dammini* trapped in New York state,[f] 2 from *I dammini* trapped in Wisconsin,[g] 1 isolate from human skin,[g] 1 isolate from the lung of an experimentally infected cat,[h] and the B-31-type strain.[i] Other spirochetes tested included *B anserina*,[j] *B turicata*,[j] *B coriacae*,[i] and 6 serovars of *Leptospira interrogans*: *bratislava*, *canicola*, *grippotyphosa*, *hardjo*, *icterohaemorrhagiae*, and *pomona*.[k]

Borrelia spp were maintained in BSK II medium at 33 C. All *Leptospira* serovars were maintained in Lepto medium[l] at 33 C. Spirochetes were enumerated in a Petroff-Hauser counting chamber. All *B burgdorferi* isolates, other *Borrelia* spp, and the 6 *L interrogans* serovars were extracted and tested by PCR analysis as described for the sample cultures.

Tissues (2-mm cubes) were digested for 8 to 12 hours in 100 µl of digestion buffer (50 m*M* Tris pH 8.5, 1 m*M* EDTA, 0.5% Tween 20, 9.43 ml of H_2O) containing 200 µg of proteinase K/µl at 37 C. The tissues were incubated at 95 C for 8 minutes, then were frozen at −70 C. They were then extracted. The PCR samples from the control and SI females and the pups from each were given a number at the end of the study and samples were assayed without operator's knowledge of the sample origin.

Known spirochete-containing cultures and blood cultures were prepared by centrifuging 1 ml of culture (12,000 × g, 30 minutes, 20 to 22 C) and extracted.[19] Tissue digests also were extracted. As carrier, yeast tRNA (0.1 mg/ml) was added prior to DNA precipitation. Pellets were air-dried, then resuspended in 20 µl of K buffer (1× PCR buffer, 0.45% Nonidet P-40, 0.45% Tween 20).[e]

Oligonucleotide primers were prepared by an automated DNA synthesizer[m] and were used without further purification. The primers were specific for DNA sequences contained in the *B burgdorferi*[20] outer surface protein (Osp A) and were designated as follows: Osp-A1, a 23-mer with sequence of 5′-AAG TAC GAT CTA ATT GCA ACA GT -3′, and Osp-A2, a 24-mer with sequence of 5′-GTT TTG TAA TTT CAA CTG CTG ACC -3′. This primer pair has been described and produces a product 646 base pairs (bp) long.[21]

Polymerase chain reaction was performed by adding 10 µl of resuspended DNA to a reaction mixture containing 1× PCR buffer (10 m*M* Tris HCl pH 8.3, 50 m*M* KCl; 3 m*M* $MgCl_2$, and 0.01% bovine serum albumin); dATP, dCTP, and dGTP[n] (200 m*M* each); 400 µ*M* dUTP[e]; 10% glycerol; 0.5% Tween-20,[e] Osp-A1 and Osp-A2 (200 m*M* each); and 1 unit of *Taq* DNA polymerase.[o] Samples were overlaid with 25 µl of mineral oil to prevent evaporation. Thermocycling was done by an automated thermocycler[p] and proceeded as follows: denature for 45 seconds at 94 C, anneal for 45 seconds at 55 C, and elongate for 2 minutes at 72 C for 45 cycles. The final cycle had an additional 8-minute incubation at 72 C to allow for completion of any unfinished elongations. The samples were kept at 4 C until stored at −20 C or until assayed on agarose gel.

To reduce the risk of amplicon carry-over contamination, the PCR samples were analyzed in a separate room from where reaction tubes were set up. Sample preparation, PCR tube set up, and sample analysis were done, using pipettors dedicated for that purpose and with plugged pipette tips. The substitution of dUTP for dTTP in the reaction mixture allowed sterilization of any possible carry-over amplified product (amplicons) contamination by using uracil DNA glycosylase[q] and an initial 37-C incubation if needed. Negative controls (water) and a *B burgdorferi*-positive control were included in every PCR assay. The BSK media and negative tissues also were tested to ensure that nonspecific amplification had not occurred.

The PCR samples were mixed with 1/5 volume of tracking dye[e] and run on a 1% SeaKem/3% NuSieve[r] agarose gel[s] at 150-V constant voltage. Gels were then stained with 10 µg of ethidium bromide/ml for 20 minutes, destained in distilled H_2O for 5minutes, and visualized by use of a UV trans-illuminator.[22,23,t]

The DNA was then transferred to a nylon membrane[u] and immobilized on the membrane, using a UV cross linker.[v] Immobilized DNA was then tested by Southern blot analysis.[24,25] Once it was determined that the amplified product was of the proper size, by using the known *B burgdorferi* isolates, only slot blotting was used.

Samples were prepared for slot blotting as described.[26] After slot blotting was performed,[w] the DNA was fixed to the nylon membrane by UV cross linking.

The probe used was a 25-mer with sequence of 5′- AGT AAC TTC CAA AGA CAA GTC ATC A -3′. It is specific for a region bound by the primers. The

[f] EBN1, EBWW, and CW1B obtained from Dr. E. Bosler, New York State Department of Health, Stony Brook, NY.
[g] MCI and WITICKI obtained from S. Callister, Gunderson Medical Foundation, LaCrosse, Wis.
[h] Obtained from Dr. E. Burgess, School of Veterinary Medicine, Madison, Wis.
[i] Obtained from the American Type Culture Collection, Rockville, Md.
[j] Obtained from Dr. Russell Johnson, University of Minnesota, Medical School, Minneapolis.
[k] Obtained from the Wisconsin State Animal Health Laboratory, Madison, Wis.
[l] Scientific Protein Laboratories, Waunakee, Wis.
[m] Applied Bio System, San Mateo, Calif.
[n] Promega, Madison, Wis.
[o] Native *Taq* Polymerase, Perkin-Elmer Cetus Corp, Norwalk, Conn.
[p] Coy Laboratory Products Inc, Ann Arbor, Mich.
[q] GIBCO BRL, Gaithersburg, Md.
[r] Nu Sieve, FMC, Rockland, Me.
[s] Mini-horizontal agarose, Submarine unit, Hoefer Instrument Co, San Francisco, Calif.
[t] Fotodyne Inc, New Berlin, Wis.
[u] Hybond N, Amersham Corp, Arlington Heights, Ill.
[v] Stratagene, La Jolla, Calif.
[w] Slot Blot PR 600, Hoefer Instrument Co, San Francisco, Calif.

forward reaction was used to end-label the probe with 5'- (γ^{32}P) ATP.[27,x] Hybridization of the labeled probe to the membrane-bound DNA was as follows: Blots were sealed in hybridization bags[y] with 10 ml of prehybridization solution of 5× Denhardt's solution (50× Denhardt's solution is: 250 m*M* Ficoll 400, 278 m*M* polyvinylpyrrolidone, 1% bovine serum albumin fraction V), 5× SSPE (20× SSPE is: 3*M* NaCl, 0.2*M* NaH_2PO_4 H_2O, 20 m*M* EDTA, pH 7.4), 0.5% sodium dodecyl sulfate (SDS), 100 μg of denatured salmon sperm DNA[c]/ml and incubated for 1 hour at 45 C. Ten microliters of the labeled probe was added to the bag, and the blot was hybridized 4 to 16 hours at 45 to 55 C. The membranes were then washed twice in 2× SSC/0.1% SDS (15 minutes at 20 to 22 C) and once in 1× SSC/0.1% SDS (30 minutes, 55 C). The blots were air-dried, wrapped in plastic wrap and exposed to film[z] between intensifying screens[aa] for 18 to 72 hours at −70 C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis—Gel electrophoresis was done according to described methods.[28] Whole-cell suspensions of passage 4 or 5 of the original inoculum strain were prepared by centrifuging the spirochetes at 12,000 × g for 30 minutes, followed by 3 washings in sterile PBSS. The pellets were suspended in sterile water, and a Lowry protein assay was done, using crystalline, bovine serum albumin (0 to 200 μg) as a standard.[29] The spirochete suspension was diluted as follows: 150 μg of protein was diluted in an equal volume of lysing agent (1.97 g of Tris HCl (0.125*M*) 4.0 g of lauryl sulfate, 20 ml of glycerol, 10 ml of B-mercaptoethanol, 0.02 g of bromophenol blue in 100 ml of water). The suspension was boiled for 90 seconds. The lysate (200 μl) was electrophoresed[28,bb] in 4% stacking gel and 12% acrylamide resolving gel (1.5 mm wide) at a constant current of 19 mA for approximately 2 hours.

Immunoblot analysis—Immunoblot analysis was done according to described methods.[28] Transfer of proteins to nitrocellulose was performed[cc] at 200 mA for 1 hour. The nitrocellulose sheets were washed 3 times in 0.1% Tween-PBSS, with constant rocking 5 min/wash. Individual strips were cut from the nitrocellulose membranes and were incubated with test sera (0.5 ml diluted 1:100 in 0.1% Tween PBSS). Each batch contained 1 strip with the SDS-PAGE molecular weight protein standards[dd] of rabbit muscle phosphorylase b (97,400), bovine serum albumin (66,200), ovalbumin (42,700), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and egg white lysozyme (14,400) for use in identifying the molecular weight of protein bands. One other nitrocellulose strip incubated with a mixture of the mouse monoclonal antibodies (H5332 directed against Osp A with an apparent molecular mass of 31 kd, and H9724 directed against the flagellar protein with an apparent molecular mass of 41 kd) diluted 1:20 was run with each batch.[20,ee] Known positive- and negative-control canine sera were assayed with each set of samples. The strips were incubated at 25 C for 2 hours with constant rocking and were washed 3 times as described.[28] The strips were then incubated for 1 hour in conjugate (horseradish peroxidase-conjugated goat anti-mouse IgG, heavy and light chain)[ff] diluted 1:500 in Tween-PBSS for the monoclonal antibodies or conjugate (horseradish peroxidase-conjugated goat anti-canine IgG, heavy and light chain)[ff] or (horseradish peroxidase-conjugated goat anti-canine IgM)[ff] diluted 1:1,000 in Tween PBSS for the canine sera. The strips were washed 3 times in 0.1% Tween-PBSS and once in PBSS, each for 5 minutes. Protein bands on the strips were developed by addition of a freshly prepared solution of 0.05% (w/v) 4-chloro-1-naphthol,[dd] 0.015% hydrogen peroxide, and 20% methanol in PBSS. The reaction was stopped by washing with cold distilled water.

Indirect fluorescent antibody test—Sera also were tested for *B burgdorferi* antibodies by the IFA test,[30] using conjugates (fluorescein isothiocyanate-conjugated goat anti-canine IgG, heavy and light chain, or fluorescein isothiocyanate-conjugated goat anti-canine IgM)[ff] diluted 1:40 in PBSS. The whole-cell antigen was the same spirochete used for inoculation. Twofold dilutions of sera were made in PBSS from 1:32 (5 log 2) to 1:1,024 (10 log 2), and the highest dilution with specific fluorescence was considered the end point. Preinoculation sera and control canine sera were used as negative controls and the same known *B burgdorferi*-positive canine serum used for the immunoblot was used as a positive control. Titer ≥ 1:128 (7 log 2) was considered a positive result.[31] Only sera from control pups and from inoculated pups that did not receive colostrum were tested for IgM antibodies, so there would be no interference of colostral antibodies.

Statistics—Litter size, birth weight, and duration of gestation were compared between the inoculated and control groups, using the Student two-tailed *t*-test (the criterion for significance using this test was $P < 0.05$).[32] The number of females that whelped per number bred, the number of females that had difficult delivery (dystocia), and the number of females that had more implantation sites than pups delivered, were compared, using Fisher's exact test (the criterion for significance, using this test, was $P < 0.05$).[33]

Results

Tissues from 3 SI females and pups from 8 of 10 litters were PCR- and/or culture-positive (Table 1; representative slot blot Fig 1). Results of blood culture for all SI females and pups were negative. All

[x] Amersham Corp, Arlington Heights, Ill.
[y] K-Pak, Minneapolis, Minn.
[z] Cronex 7L, DuPont, Wilmington, Del.
[aa] Lightning Plus, DuPont, Wilmington, Del.
[bb] Mighty Small II, Hoefer Scientific Instruments, San Francisco, Calif.
[cc] TE Mini-transfer unit, Hoefer Scientific Instruments, San Francisco, Calif.
[dd] Bio-Rad Laboratories, Richmond, Calif.
[ee] Obtained from Dr. A. Barbour, Department of Microbiology, University of Texas Health Science Center, San Antonio.
[ff] Kirkegaard & Perry Laboratories, Gaithersburg, Md.

Table 1—Results of polymerase chain reaction (PCR) test and culture results of tissues from spirochete-inoculated females 1-10 and their pups, number of pups *B burgdorferi*-positive per litter, and age at death of such pups

Female	Positive maternal tissues	Total No. positive*	Tissues positive by PCR	Age atdeath	Culture-positive samples
1	None	3/3	P1 Kidney, heart, spleen, lung	P1 3 d	P1 Urinary bladder,† kidneys, heart,
			P2 Liver, lung	P2 5 d	spleen, thymus, liver
			P3 Spleen, lung, heart	P3 7 d	P2 Thymus,† heart†
2‡	None	0/0	NA	NA	NA
3	None	2/5	P1 Brain	P1 Stillborn	
			P2 Thymus, heart, brain	P2 30 min	NA
4	Spleen	3/7	P2 Bladder	P2 2 d	
			P4 Thymus, brain, liver	P4 6 wk	
			P5 Lung, spleen, lymph nodes	P5 6 wk	NA
5	Lymph node, spleen	6/7	P1 Brain, kidney, thymus	P1 6 wk	Lymph node of female
			P2 Bladder	P2 6 wk	NA
			P3 Brain	P3 6 wk	NA
			P4 Brain, liver, lymph node, bladder	P4 6 wk	NA
			P5 Lung, brain, thymus	P5 6 wk	NA
			P6 Lung	P6 6 wk	NA
6§	None	0/0	NA	NA	NA
7	None	1/6	P3 Brain, kidney	P3 6 wk	NA
8	Brain, lymph node	1/5	P4 Liver	P4 4 d	NA
9	None	1/3	P1 Lung, kidney, heart	P1 6 wk	NA
10	Bladder	2/5	P3 Spleen	P3 6 wk	P4 Liver
			P4 Liver	P4 1 d	

* No. of pups PCR-positive/total pups in liter. † Tissue not tested by PCR—insufficient amount. ‡ Never underwent estrus. § Bred, but never became pregnant.
P = Pup No.; NA = not applicable.

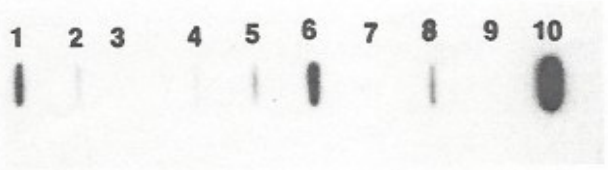

Figure 1—Slot blot of tissue samples from pups (P) of spirochete-inoculated females. Tissues from pups of female 1: lane 1 = P1 kidney, lane 2 = P1 spleen; from female 3: lane 4 = P1 brain, lane 5 = P2 thymus; from female 7: lane 6 = P3 brain; from female 8: lane 8 = P4 liver. Lanes 3 and 7 are brains from control pups, lane 9 is a water control, and lane 10 is a positive control.

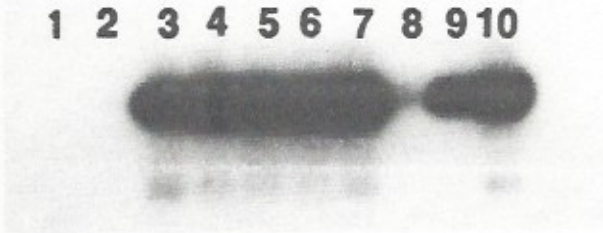

Figure 2—Sensitivity of polymerase chain reaction (PCR) analysis. *Borrelia burgdorferi* (EBN1) was cultured in BSK II medium, and spirochete concentrations of 10^6 to 10^0 cells/ml were then amplified in 45 cycles of annealing temperature of 55 C and Mg^{2+} concentration of 3 m*M*. The BSK II medium and water were used as controls. The PCR products were size-fractionated on a 3% NuSieve, 1% SeaKem agarose gel, blotted, hyridized, and autoradiographed. Lane 1 = water control, lane 2 = BSK II medium, lane 3 = 10^6 spirochetes/ml, lane 4 = 10^5/ml, lane 5 = 10^4/ml, lane 6 = 10^3/ml, lane 7 = 10^2/ml, lane 8 = 10/ml, lanes 9 and 10 = EBN1-positive control.

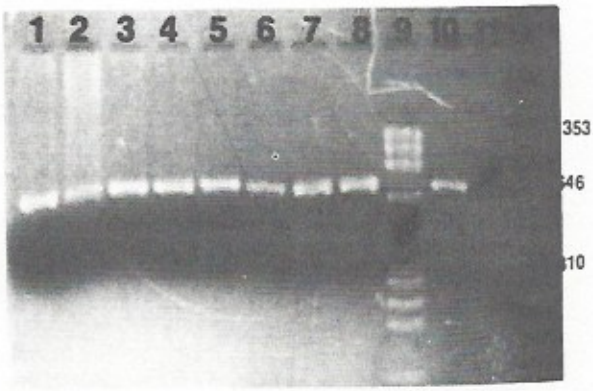

Figure 3—Specificity of PCR analysis of *B burgdorferi* isolates. Isolates were cultured in BSK II medium, then were amplified in 45 cycles withy annealing temperature of 55 C and Mg^{2+} concentration of 3 m*M*. The BSK II medium and water were used as negative controls. The PCR products were size-fractionaed on a 3% NuSieve, 1% SeaKem agarose gel. Molecular weight of some of the marker fragments is indicated in base pairs on the right. Lane 1 = tick isolate EBN1, lane 2 = tick isolate EBWW, lane 3 = tick isolate CW1B, lane 4 = Wisconsin tick isolate MC1, lane 5 = Wisconsin tick isolate WI TICK1, lane 6 = human skin isolate, lane 7 = cat isolate, lane 8 = B-31 isolate, lane 9 = marker $\phi\times$ 174 digested with *Hae* III, lane 10 = EBN1, lanes 11 and 12 = negative controls, water and culture medium, respectively.

tissues and blood from the control females and their pups were culture- and PCR-negative.

The PCR test was shown to be sensitive and specific in detecting *B burgdorferi* DNA from spirochetes cultured in BSK II medium. Using agarose gel electrophoresis and Southern blotting, < 10 organisms/ml could be detected (Fig 2). The 8 *B burgdorferi* isolates were directly amplified from culture to determine the specificity of this PCR. All 8 strains had amplification products of the correct size (Fig 3). None of the other *Borrelia* spp or the 6 *Leptospira* serovars had amplification products (data not shown). The PCR appeared to be specific for *B burgdorferi* in tissues, as evidenced by the fact that all samples from controls (10 adult females and 56 pups) were *B*

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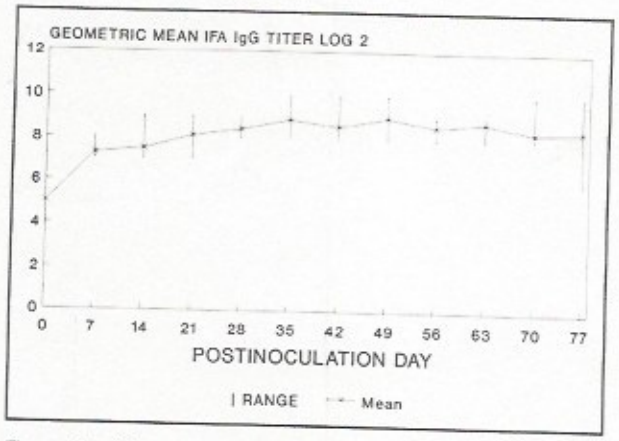

Figure 4—Mean and range of the indirect fluorescent antibody (IFA) test results for the 10 females inoculated with *Borrelia burgdorferi*. Results are given as log 2 of the highest dilution with specific fluorescence.

burgdorferi-negative and only tissues from SI females and their pups were *B burgdorferi*-positive.

Serologic responses of the 10 SI females were determined by the mean value of the IFA test (Fig 4) and by WB results. Titer for the IFA was expressed as log 2, so it can be expressed as a mean value. All 10 SI females had a fourfold or greater increased antibody titer over preinoculation values. None of the 10 control females had titer increase greater than preinoculation titer of 5 log 2 (1:32). Of 10 inoculated females, 9 developed IFA IgG titer ≥ 6 log 2 (1:64) by week 2 after inoculation, and the remaining female (no. 3) had this titer by week 3 after inoculation. Peak titers were 9 log 2 (1:512, No. 1, 3, 5, and 7) and 10 log 2 (1:1,024, No. 2, 4, 6, 8, 9, and 10). All SI females developed IgG antibodies, as measured by WB, that reacted to *B burgdorferi* species-specific proteins Osp A (31 kd) and Osp B (34 kd), and females 5 and 7 had antibodies to the species-specific 39-kd protein (Fig 5). In addition, females 1, 2, 4, 5, 7, and 8 developed antibodies to the genus-specific flagellar protein (41 kd), females 8 and 10 also developed antibodies to proteins in the 60- to 70-kd range (Fig 6). All control females remained *B burgdorferi*-negative by IFA and WB results until they were euthanatized.

For pups (reciving colostrum) of individual litters from the SI females, serologic test results reflected the titer of the bitch at whelping. Pups receiving colostrum (litters from females 1, 5, 7, 8, 9, and 10) had IgG antibodies to *B burgdorferi* detected by the IFA test or WB and had the same titer as the bitch on the day of whelping (Table 2). These titers decreased or results became negative by 6 weeks. These findings indicate that the antibodies in the pups were maternally derived.

Pups from litters 3 and 4 did not receive colostrum because of dystocias and lack of milk let-down. Litter 3 comprised 5 pups: 2 were stillborn, 2 were alive, and 1 was found in the uterus at necropsy; the dam was euthanatized during delivery because of complications. The stillborn and live pups were seronegative for IgG antibodies to *B burgdorferi* by the IFA test and WB; serum was not obtained from the pup in the uterus. Sufficient serum was not available from these pups to test for IgM antibodies. Because the 7 pups from female 4 did not receive colostrum (no colostral antibodies), their sera were tested for IgG and IgM antibodies. At birth, 7 pups were seronegative for IgG and IgM by the IFA test and for IgG by WB, and remained so until 6 weeks of age. The IgM antibodies to the 34- and 22-kd proteins were detected by WB in pups 2, 5, 6, and 7 at birth and by day 7. All but pup 7 that died at 3 days of age, also had antibodies to the 41-kd protein. These antibodies persisted until 6 weeks of age. Pups 1, 3, and 4 were negative for IgM antibodies by WB on day 1, but by day 7, they developed antibodies to the 34- and 41-kd proteins, which persisted until 6 weeks of age. Antibodies to the 22-kd protein developed in pups 4 and 5 by week 4 and persisted until 6 weeks. All control pups were seronegative for IgG and IgM antibodies by WB and the IFA test at day 1 and remained so until 6 weeks of age.

Of 9 SI females that were bred, 8 carried their litter to term. One female was not bred, and 1 was bred, but did not become pregnant. Spirochete-inoculated female 2 began proestrus on 3 separate occasions 45 days apart, but never progressed to estrus on any occasion. She was euthanatized after the third failure to reach estrus and was necropsied. Spirochete-inoculated female 6 was bred similarly as the

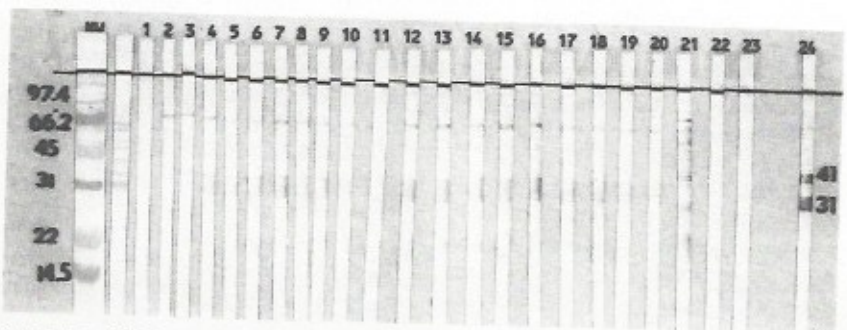

Figure 5—Protein immunoblot of IgG antibodies to *Borrelia burgdorferi* in spirochete-inoculated female 10. Serum was obtained weekly from day 0 to postinoculation week 19. Lanes represent the following: MW = molecular weight markers, unmarked lane = *B burgdorferi* protein profile stained with Ponceau S, lanes 1–20 = weekly serum from day 0 to week 19, lane 21 = positive-control canine serum, lane 22 = negative-control canine serum, lane 23 = phosphate-buffered [illegible]

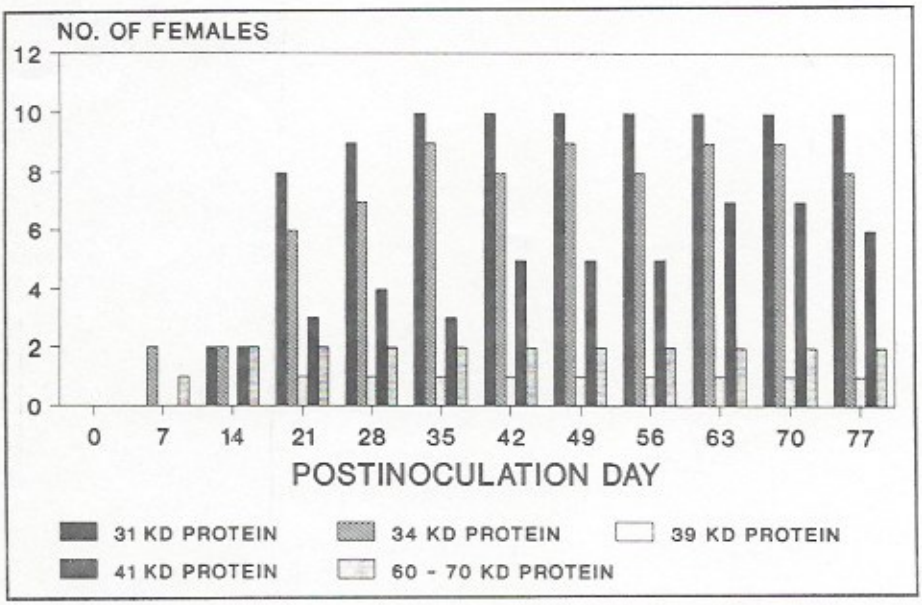

Figure 6—Summary of the western blot analysis results of the 10 females inoculated with *Borrelia burgdorferi* (days 0–77). Results are given as antibodies to be 31-, 34-, 39-, 41-, and 60- to 70-kd range of *B burgdorferi* proteins.

Table 2—Indirect fluorescent antibody (IFA) and western blot (WB) results of sera tested for IgG antibodies to *Borrelia burgdorferi* from the spirochete-inoculated females on the day of whelping and from their pups on days 1 and 42 of age

	Female No.							
Animal	1	3	4	5	7	8	9	10
Females								
IFA*	128	256	256	Neg	128	Neg	128	1,024
WB†	31, 34	31, 34	31, 34	31, 34	31, 34	31, 34	31, 34	31, 34, 41
Pups‡								
Day 1								
IFA	128, (3/3)§	Neg (4/4)	Neg (7/7)	Neg (7/7)	128 (2/6)	Neg (5/5)	128 (1/3)	1,024 (5/5)
WB	31, 35 (3/3)	Neg (4/4)	Neg (7/7)	31, 34 (7/7)	Neg (4/6)	31, 34 (5/5)	Neg (2/3)	31, 34, 41
					31, 34 (4/6)		31, 34 (3/3)	(5/5)
					Neg (2/6)			
Day 42								
IFA	NT‖	NT‖	Neg (6/7)¶	Neg (7/7)	Neg (6/6)	NT‖	128 (1/3)	128 (5/5)
WB	NT	NT	Neg (6/7)	Neg (7/7)	Neg (6/6)	NT‖	Neg (2/3)	31, 34, 41
							31, 34 (3/3)	(5/5)

* Given as the reciprocal of the highest dilution with specific fluorescence. † Given as antibodies to the 31-, 34-, 41-, or 22-kd proteins. ‡ Pups of females 1, 5, 7, 8, and 10 received colostrum prior to day 1 sample collection. Pups of females 3 and 4 were not given colostrum. Females 2 and 6 did not have a litter. § No. of pups with result/total no. of pups. ‖ Died prior to day 42. ¶ One died prior to day 42.
NT = not tested. Neg = negative.

others, but did not become detectably pregnant. This female was euthanatized 6 weeks after the projected whelping date.

Mean litter size, mean birth weight, number whelped per number bred, duration of gestation, dystocias, mortality, and information on uterine implantation sites of control and SI females were compared (Table 3). Total pup mortality for the control group of females was 21%, compared with overall mortality of the original commercial Beagle colony (from which the dogs were purchased) of approxiamtely 25% from birth to 6 weeks of age. Of the 12 pups that died before 6 weeks in the control group, 9 were from 2 females. These pups died within the first 12 hours. Each of these females lost all but 1 pup from their respective litter. Similar findings were observed in the original colony, that most pup mortality was associated with few females. Of the pups delivered in the SI group, 37.5% died before 6 weeks of age. Of this group, 13 of the 15 pups that died were from 3 females, all of which lost their entire litter. Because mortality was not uniform throughout all litters, statistics were not applied to these data. These females, however, lost their litter for various reasons. Spirochete-inoculated female 3 lost all pups because of complications resulting from dystocia; SI female 1 lost its litter because of bacterial infection. A β-hemolytic *Streptococcus* spp was isolated from organs in 2 of 3 pups and from the vagina of the female. Spirochete-inoculated female 8 lost its litter because of poor maternal care. Pup 4 from SI female 10 died 1 day after delivery and pup 2 from SI female 4 died 2 days after delivery.

Differences between the 2 groups in litter size, birth weight, or number whelped per number bred were not significant. On the basis of more implantation sites than pups delivered ($P = 0.086$), there was a possibility that SI females resorbed fetuses. None of the control fetuses were lost throughout their gestation period, as determined by ultrasonography and by evaluation of placental attachment sites. When the 2 groups were compared for complications in deliv-

Table 3—Comparison of litter size, birth weight, number bred per number whelped, expected whelping date, ease of delivery, pup mortality, and number of uterine implantation sites in control and spirochete-inoculated females

Females	Litter size	Birth weight	No. whelped/ No. bred	Average gestation duration	Ease of delivery	Mortality by 6 weeks of age	Uterine implantation site
Control	6 ± 0.65	0.31 kg ± 0.001	10/10	63.5 ± 0.82	No observable problems	21.6%	Same number of sites as pups delivered
Inoculated	5 ± 0.65	0.295 kg ± 0.001	8/9	66.5 ± 0.82	3/8 dystocias*	37.5%	3/9 dogs bred had more sites than pups delivered

* Female 3 was euthanatized while delivering pup 4, because of traumatic complications; females 4 and 9 had dystocia, delivering 4 days after their calculated due date. † Female 10—6 implantation sites, 1 incompletely resorbed fetus—5 pups delivered; female 9—6 implantation sites, 3 pups delivered; female 6—6 implantation sites, no pups delivered.
Data are reported as mean ± pooled SEM.

ery, the SI group had a tendency to have more dystocias ($P = 0.06$). These dystocias may be related to increased mean gestation duration in this group. The mean gestation duration for the infected group was 3.5 days beyond the predicted gestation duration of 63 days. Comparison of mean gestation duration of both groups (t value of 3.614, df 16) indicated a difference between the 2 groups ($P < 0.01$).

None of the control of SI females developed clinical signs of disease, (lameness, stiffness, or anorexia) throughout their gestation period or in the 6-week postpartum period. Gross or histologic lesions were not observed in any of the control or SI females or pups.

Discussion

Intrauterine infection of pups with *B burgdorferi* was detected after intradermal inoculation of females on the basis of spirochete isolation and detection of *B burgdorferi*-specific DNA in tissue by PCR in experimentally infected pregnant Beagles and pups. The intradermal route of inoculation has been documented to be effective, even with as low a dose as 100 organisms.[34] We chose to infect at 2-week intervals, in an attempt to maximize the chance of infection. Dogs living in endemic areas may be exposed to *B burgdorferi*-infected ticks for several months and ticks could feed on them repeatedly during gestation. The SI females did not manifest clinical signs or infection during gestation, but a suggestion of increased dystocias and fetal resorptions was apparent. Their pups became infected, but they did not manifest clinical signs of infection, inflammatory response, or increased mortality.

All 8 SI females that had litters delivered pups in which at least 1 had PCR-detectable *B burgdorferi* DNA, including 4 pups < 2 days old (1 stillborn pup and 1 that died at 30 minutes of age from SI female 3, a 1-day-old pup from SI female 10, and a 2-day-old pup from SI female 4), providing evidence of intrauterine transmission. These findings reflect maternal systemic infection and ability of the spirochetes to infect fetuses in the uterus. It is not possible for these pups to have a disseminated infection of the tissues, because in a previous study, it took a minimum of 3 days after inoculation for spirochetes to invade multiple tissues.[35] *Borrelia burgdorferi* DNA was detected in tissues of 16 other pups from SI females, but it could not be determined whether infection was before or after parturition because the pups were > 3 days old. Of 7 pups from SI female 4 that had not received maternal colostrum, 3 had IgM antibodies to *B burgdorferi* proteins within the first 24 hours after parturition. Presence of IgM antibodies indicates possible primary immune response of the pups because IgG is the predominant immunoglobulin class that is transferred maternally.[36] It is possible that exposure of the immature immune system in the fetuses to low numbers of spirochetes could cause incomplete tolerance to *B borgdorferi* proteins. The higher affinity-B cells that produce IgG are more susceptible to tolerance than those producing IgM. Persistent stimulation to the immune system would continue to stimulate further production of IgM. This could explain why colostrum-deprived pups continued to produce IgM without the development of IgG through 6 weeks after parturition.[37] Two of these pups had PCR-detectable *B burgdorferi* DNA in their tissues.

All 8 SI females that had litters became infected with *B burgdorferi* antibodies detectable by the IFA and WB and by detection of *B burgdorferi* by PCR and/or spirochete culture in the tissues of either the females or pups in their litters.

One SI female had a culture-positive lymph node, indicating either persistent or repeated reinfection. The *B burgdorferi*-specific DNA sequences in tissues of 6-week-old pups could indicate either intrauterine infection or contact transmission[23] from the infected dam. The absence of spirochetes in tissues of some pups from litters of SI females in which at least 1 pup was infected, may indicate that the organisms were present in numbers too small to be detectable by our PCR system or an inability of the spirochete to infect all pups or tissues equally.[38]

The discrepancy between the presence of PCR-detectable *B burgdorferi* DNA in tissues and inability to isolate the organism from the same tissues, in most instances, may be attributable to the difficulty in isolating the spirochete in culture or that the spirochetes were no longer viable and only spirochetal DNA was in the tissues. Polymerase chain reaction has been shown to be more sensitive than culture.[39] Inapparent spirochetosis has been observed in fetuses from clinically normal cows infected with *B coriacae*[40] and this may have been the case in the infected pups.[38] The lack of inflammatory reaction in infected pups was similar to the lack of response to

transplacental infection of *B burgdorferi* in a human fetus.[11,14] It was thought that lack of inflammatory response in the human fetus and neonate was attributable to an immature immune system.[11,14] Although we were not able to associate mortality with *B borgdorferi* infection in this study, transplacental transmission of other spirochetes (relapsing fever and leptospirosis in human beings) has been reported and has been associated with increased fetal loss.[41,42]

Intrauterine infection with *B burgdorferi* is a mechanism by which pups can become infected in the absence of a vector. Furthermore, 6-week-old pups that were delivered by an infected female had *B burgdorferi*-specific DNA detected in their tissues. Whether this was a result of contact or intrauterine transmission is unknown. It does, however, indicate that the spirochete may have been transmitted in the absence of a tick vector. These findings represent a potential means of transmitting *B burgdorferi* in a population of dogs, even though ticks may be absent. *Borrelia burgdorferi* infection should be included in the differential diagnosis of reproductive failure in dogs, especially if prolonged gestation periods and stillbirths are observed. Further investigations are necessary to elucidate the clinical manifestations of *B burgdorferi* infection in pregnant dogs, and the intrauterine and postparturient effects, short-term and long-term, that such infections would have on pups.

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