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VIII Annual Scientific Conference on Lyme Borreliosis
Guest Editor: *to be announced*

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GUEST EDITORIAL

Lyme borreliosis has been reported in dogs, cats, horses, cattle and sheep. Both nymphal and adult *Ixodes* species usually transmit *Borrelia burgdorferi* to domestic animals. Even though other species of ticks and hematophagous insects including fleas have been shown to harbor the bacterium, their involvement in pathogen transmission has not been determined. Contact transmission has been reported in dogs and *B. burgdorferi* shedding in urine of cattle has been demonstrated.

A large percentage of domestic animals in endemic areas are apparently asymptomatically infected with *B. burgdorferi* and seroconvert while appearing clinically normal. In general, domestic animals with clinical disease primarily exhibit single or migratory lameness and joint swelling that is variably accompanied by fever. Susceptibility and expression of clinical illness seems to vary both between species and individuals. A variety of other clinical signs have been less frequently reported in domestic animals including behavioral changes, seizures, encephalitis, renal dysfunction, cardiac arrhythmia and reproductive disorders.

Diagnosis of disease in veterinary medicine suffers from many of the same problems and inadequacies experienced in human medicine and is further exacerbated by the inability to directly communicate with the patient. Presumptive diagnosis depends on recognition of clinical signs, ruling out of other causes of lameness and joint swelling, supportive serology (indirect immunofluorescence and enzyme-linked immunosorbent assays and immunoblot), and response to antibiotic therapy. Serological evidence alone is not sufficient to support a diagnosis of Lyme disease in domestic animals due to the large number of infected animals which appear clinically normal. Definitive diagnosis relies on recovery and identification of the organism from affected tissues by various methods including immunostaining, silver staining, in vitro cultivation and polymerase chain reaction. As in human medicine, improved diagnostic tests are needed. Treatment regimens have been largely extrapolated from laboratory animal studies and human patient experience and no regimented drug trials have been conducted on domestic animal species. Antibiotics in the penicillin and tetracycline familes are typically employed with drugs and modalities varying between species.

Lyme borreliosis has been investigated more thoroughly in dogs than in other domestic species. Serological estimates of canine exposure to *B. burgdorferi* range from 40–89 percent in endemic areas while clinical disease develops in only about 5 percent of the exposed dogs. Dogs with clinical illness most commonly present with acute onset of single or shifting limb lameness, swollen joints, fever and depressed attitude. A less common presentation is that of recurring single-joint lameness with low grade fever that possibly represents a more chronic infection. Radiographic findings of the most commonly affected joints (carpus, elbows, tarsus) are unremarkable. Other less commonly observed manifestations of Lyme borreliosis in dogs include myocarditis, renal failure and neurological disorders including behavior changes and seizures. These presentations parallel findings in human medicine and indicate the disease in dogs is also multisystemic.

Several laboratories have experimentally infected dogs in an attempt to develop a canine model to study pathogenesis of Lyme borreliosis and for evaluation of preventative and therapeutic treatment procedures. Dogs infected by inoculation of organisms did not develop clinical signs with the exception of one challenge model that utilized repeated inoculations of *B. burgdorferi* into dexamethasone treated dogs. In this study *B. burgdorferi* was cultivated from blood of infected dogs and clinical signs observed consisted of lameness, fever and behavioral changes.

More recently canine models that more closely mimic natural disease have been developed using *B. burgdorferi* infected ticks as the source of infection. In one model described by Dr. Appel and coworkers dogs seroconverted 4 to 6 weeks following infection, developed intermittent lameness 2–4 months after infection and had persistent elevated antibody titers for more than a year following infection. Lesions were restricted to joints, lymph nodes and skin and no signfiicant lesions were seen in any other tissues examined. Histopathology of joints of infected dogs with acute lameness showed a fibrinopurulent arthritis and synovitis while joints of clinically normal dogs that had seroconverted showed a mild nonsuppurative plasma cell and lymphocytic infiltrate of synovial membrane and joint capsule. *B. burgdorferi* was isolated most frequently from skin at tick bite sites and from adrenal and muscle tissue.

Clinical, serological and histopathological findings from a second laboratory in which beagles were naturally infected by ticks are presented in this issue. Although infection in this model was established by cultivation of *B. burgdorferi* from ear punch biopsies two weeks after tick feeding, clinical evidence of disease was limited to positive serology, mild gait abnormalities and cyclic aggressive behavior. Increased aggression and lameness appeared six months following initial exposure to infected ticks and continued intermittently for nine months. There was little gross or microscopic pathology associated with infection by *B. burgdorferi* even though organisms were cultivated from the urinary bladder in one dog and from the bladder, meninges and thyroid from a second dog.

Clinical signs observed in both models of natural infection in immunocompetent dogs correlate very well with reports of naturally exposed populations and demonstrate the difficulty of using a single set of clinical criteria for diagnosing subacute or chronic Lyme borreliosis in dogs.

Another article in this issue deals with detection of *B. burgdorferi* antigen in urine. Although viewed primarily as a diagnostic tool in human medicine, urine shedding of *B. burgdorferi* in white-footed mice and cattle may be part of an alternative route of transmission. Live organisms have been cultured from the urine of naturally infected white-footed mice and both naturally and experimentally infected cattle. Even though *B. burgdorferi* cannot survive freely in the environment, the behavior patterns of freely housed cattle in which direct contact of urine to the nasal, oral and conjunctival mucous membrane is common suggests urine-mucous membrane transmission of this organism is possible. These facts force us to broaden our view of how this disease may be maintained in nature.

Sandra L. Bushmich, M.S., D.V.M. and Edward M. Bosler, Ph.D.

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SPECIAL EDITORIAL
Happy 70th Birthday to Willy Burgdorfer—Discoverer of the Lyme Disease Agent

Tom G. Schwan

Most of us who have been impacted in some way by Lyme borreliosis, whether through vocation or affliction, are aware that the causative agent of this disease was discovered by Dr. Willy Burgdorfer. However, this tremendously important discovery came relatively late in his career, following many other significant contributions of which many people might not know. Therefore, it seems appropriate as we honor his 70th birthday to look back at many of the other accomplishments of this outstanding researcher and fine human being.

Dr. Burgdorfer was born in Basel, Switzerland, on June 27, 1925, where he grew up and received all of his formal education. In 1951, he completed his Ph.D. in parasitology and tropical bacteriology under the guidance of Professor R. Geigy, including a thesis investigating the dynamics of the African relapsing fever spirochete, *Borrelia duttonii*, in its tick vector, *Ornithodoros moubata*. His early training and research with pathogenic spirochetes would later provide Dr. Burgdorfer with the skills allowing him to discover the causative agent of Lyme borreliosis 30 years later! Upon completion of his Ph.D., Dr. Burgdorfer received a U.S. Public Health Service fellowship and went to the Rocky Mountain Laboratory (RML) of the National Institute of Allergy and Infectious Diseases in Hamilton, Montana, in 1951, where he actively researched a variety of arthropod-borne disease agents, spanning 35 years until his retirement in January 1986.

During his tenure at RML, he was Research Entomologist (1957 through 1972), Head of the Rickettsial Diseases Section (1973 through 1978), Head of the Arthropod-borne Diseases Section (1979 through 1982), and Acting Chief of the Epidemiology Branch (1982 through 1985). When Dr. Burgdorfer retired in 1986, he was awarded the status of Scientist Emeritus by the National Institute of Allergy and Infectious Diseases, an honor given to only a few NIH scientists for outstanding accomplishments and scientific contributions made during their careers. Since his retirement in 1986, Dr. Burgdorfer has continued an active program combining research, consulting, writing, and numerous invitational speaking engagements. Dr. Burgdorfer's ability to speak and write in English, German, and French makes him unique in bridging the gap between North American and European scientists.

Dr. Burgdorfer has authored or coauthored over 200 scientific publications during the last 46 years (1949 through 1995). Through his research and scientific writings, he has become an internationally recognized expert on tick-borne pathogens, especially rickettsiae and borreliae. Dr. Burgdorfer, however, also made pioneering discoveries on the persistence of Colorado tick fever virus in the red blood cells of mammals infected with this virus, he discovered Snowshoe hare virus, and he described the phenomenon of inhibition of infection of virulent spotted fever rickettsiae in ticks by the presence of nonvirulent rickettsiae. Dr. Burgdorfer also developed the hemolymph test to assay live ticks for rickettsial infection and demonstrated the usefulness of staining the hypodermis of dead ticks for detecting these bacteria. Both assays are used worldwide for studying the distribution of tick-borne rickettsiae.

In late 1981, Dr. Burgdorfer discovered spirochetes in the midguts of *Ixodes scapularis* ticks sent to him at RML from Shelter Island, New York. Although looking for rickettsiae, Dr. Burgdorfer's years of research on African and American tick-borne relapsing fever spirochetes allowed him to succeed after many others had failed. He had discovered the causative agent of Lyme disease and, by doing so, has opened up entirely new areas of research in medical entomology, bacteriology, clinical medicine, and the list goes on. In a way, many of us owe our jobs to Dr. Burgdorfer's discovery. In 1984, the taxonomic description of the Lyme disease spirochete was published by Russell Johnson and coworkers. The spirochete was named *Borrelia burgdorferi*, honoring Dr. Burgdorfer for his seminal discovery.

For both his years of significant contributions toward our understanding of tick-borne pathogens and his discovery of the Lyme disease agent, Dr. Burgdorfer has received numerous honors. Some of his awards include a Guggenheim Fellowship (1984); a Department of Health, Education, and Welfare Superior Science Award (1974); the Schaudinn–Hoffman Plaque from the German Society of Dermatology (1985); an Honorary Medical Degree from the University of Bern, Switzerland (1986); The Robert Koch Gold Medal from the Robert Koch Foundation, Berlin, Germany (1988); the Bristol Award, which is the highest honor granted by the Infectious Diseases Society of America (1989); honorary degrees from the University of Montana (1990) and The Ohio State University (1994); and the Walter Reed Medal from the American Society of Tropical Medicine and Hygiene (1990).

Dr. Burgdorfer is also an honorary life member of the American Society of Rickettsiology (1986) and the International Northwestern Conference on Diseases in Nature Communicable to Man (1987) and a past president of the American Society of Rickettsiology (1982). Dr. Burgdorfer has participated in numerous scientific national and international committees, and he is currently on the Board of Directors of the Lyme Disease Foundation.

Outside the laboratory, Dr. Burgdorfer has led an exemplary life. He and his wife, Dale, raised two fine sons, and he has served the community of Hamilton through his activities with both the church and Kiwanis International. He is a true gentleman, kind, and always willing to give and share his time whether in his office or at home.

Dr. Burgdorfer will be 70 years old this June, and he continues to be an active member of the scientific community. His

accomplishments and discoveries are second to none, and few scientists—past, present, or future—have made or will make discoveries that impact on so many areas of biomedical research. It is a pleasure to lead the Lyme Disease Foundation in wishing Willy a very happy 70th birthday, and I hope there are many more to come as we continue to enjoy his presence and friendship and benefit from his assistance.

Tom G. Schwan, Ph.D.
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Canine Lyme Borreliosis I. Gross Clinical Observations of Laboratory Beagles following Exposure to Ticks Infected with *Borrelia Burgdorferi*

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Virginia-Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia (R.D.E., J.L.R.), New York State Department of Health, Health Sciences Center, Stony Brook, New York (E.M.B.), Intervet International BV, Boxmeer, The Netherlands (F.O.), Veterinary Research Associates, Farmingdale, New York (E.M.S.), Department of Veterinary Microbiology, University of California, Davis, California (R.B.L.), and Intervet Inc., Millsboro, Delaware (M.D.G.)

Lyme borreliosis in dogs has been reported to be associated with lameness, fever, lethargy, lymphadenopathy, heart block, polyarthritis, renal lesions, and neurologic changes. Attempts to produce laboratory models of the disease have had limited success and have indicated that the disease either is difficult to reproduce in laboratory studies or is only mildly evident in dogs.

Ten mated pairs of *Borrelia burgdorferi*–infected adult *Ixodes scapularis* ticks were allowed to feed to repletion on laboratory beagles. Following tick detachment, a new set of unfed adult ticks were placed on the beagles and again allowed to feed to repletion. Clinical signs were recorded and scored daily. Blood and cerebrospinal fluid samples were collected for serological analysis. Dogs were euthanatized at the end of the observation period, and standard histological procedures were conducted.

Increased aggressive behavior and clinical signs indicative of polyarthritis began appearing approximately 6 months after initial exposure and continued in a semicyclic pattern through the 9th month. Serological response to whole-cell sonicates and specific proteins was detected in exposed dogs as early as 2 weeks post exposure. Histological evidence of disease was minimal and was limited to the kidneys and adrenal glands.

Although infection by *B. burgdorferi* was established in these dogs, clinical evidence of disease was limited to serology, mild gait abnormalities, and cyclic aggressive behavior. Infected dogs gained significantly more weight than the noninfected controls, which may be indicative of disease-induced behavioral changes.

Key words: Canine, Lyme disease, *Borrelia burgdorferi*

Lyme disease is caused by the bacterium *Borrelia burgdorferi* (Bb) and is manifested most commonly as a chronic progressive inflammatory disorder but has protean manifestations in humans (1–4). The bacterium is known to be transmitted by the bite of infected *Ixodes scapularis* ticks (5–7). Lyme borreliosis in dogs was initially reported to be associated with lameness and fever (8). Subsequent reports indicating lethargy, lymphadenopathy, heart block, polyarthritis, renal lesions, and neurologic changes suggest a correlation of the disease in dogs to the reported manifestations in man (1, 4, 8–15). Recent laboratory experiments with dogs, using immunomodulators and multiple injections of Bb, indicated that rapid onset of a severe recurring lameness, fever, inappetence or anorexia, and intermittent signs of depression could be induced in most of the exposed dogs by this method (16, 17). The rapid onset of severe clinical signs in a high percentage of the exposed animals was somewhat unusual considering the published reports of clinical observations from field situations (18–20). Dogs used in serological surveys that tested positive for Bb antibodies often had no complications, even though spirochetes were cultured from some dogs (18, 19). Appel et al. reported that clinical signs of Lyme disease were more evident in young dogs and that the pattern of disease in dogs following injection versus tick feeding was markedly different (21). The purpose of the present study was to produce natural infection in adult immunocompetent dogs and to follow the course of the disease by clinical observations for an extended period of time under controlled conditions.

*Corresponding author

METHODS

Animals and husbandry

Fourteen laboratory beagles (Marshall Farms, Rochester, NY) were housed in isolation facilities throughout this experiment. Dogs were individually caged until termination of tick feeding and positive reisolation of Bb was made. They were provided *ad libitum* access to commercial food ration in multiple trough feeders, and water was supplied by nipple waterers. Dogs were vaccinated against Canine Parvovirus, Canine Distemper, Canine Hepatitis, *Bordetella bronchiseptica*, and *Leptospira interrogans*. Serological analysis for response to these antigens indicated the ability of these dogs to respond immunologically. The dogs were randomly divided into two groups. Males were neutered prior to placing with females. One group contained four dogs (not exposed) and the other group contained 10 dogs (exposed). Randomly selected dogs, four exposed and one nonexposed, were euthanized at 9 months postexposure, and the remaining dogs were euthanized at 12 months postexposure.

Tick exposure

At 1 year of age, the dogs were subjected to tick feeding, as previously described (22). Four dogs were maintained as nonexposed controls, five were subjected to continuous tick feeding (new set of tick pairs placed 1 to 2 days following detachment of the previous set), and five were subjected to

TABLE 1
Scoring Criteria for Clinical Observations of Dogs Exposed to Ticks Infected with B. burgdorferi

Symptom	Frequency	Scoring Description
Temperature	Weekly[a]	°C
Weight	Weekly	Kg
General impression	Daily	0 = active
		1 = depressed
		2 = inappetent
		3 = inactive
Temperament	Daily	0 = normal
		1 = reclusive and defensive
		2 = aggressive
Gait	Daily	0 = normal
		1 = slight stiffness when moving
		2 = stiff, reluctant to move
		3 = limping

[a]Dogs with temperatures ≥39.5°C were monitored on subsequent days to determine the duration of elevated temperature.

TABLE 2
Categories for Evaluating General Impression and Temperament

General Impression	Temperament 0 Normal[a]	1 Reclusive/ Defensive[b]	2 Aggressive[c]
0 active	Y[d]	N	Y
1 depressed	Y	Y	N
2 inappetent	Y	Y	Y
3 inactive	Y	Y	N

[a]Dogs in this category appeared to socialize normally.
[b]Dogs in this category remained separated from the group and growled or snarled at the approach of other dogs.
[c]Dogs in this category attacked other dogs without provocation.
[d]Y = yes, a possible combination. N = no, an improbable combination.

discontinuous feeding (new set of tick pairs placed 7 days following detachment of the previous set). Feeding cycles were repeated seven times in the continuous group and five times in the discontinuous group. Confirmation of infection was made by ear biopsy, as reported, and cultural confirmation was performed as described below (22). Following positive identification of Bb in all exposed dogs (day 70 postinitial exposure), they were moved from individual caging to a single isolation room with the nonexposed dogs (day 90 postinitial exposure).

Clinical observations

Independent veterinarians and technicians certified by the American Association of Laboratory Animal Science monitored all dogs daily for clinical signs (Table 1). Observations were taken in a blind manner, as observers were not informed as to the treatment protocol for any dog. Weight, temperature, and general physical condition for each animal were recorded weekly for 6 months prior to the initiation of the study. Gait scores recorded each day were used for statistical comparison by logistic regression analysis in the SAS procedure LOGISTIC (23). Group and time were assigned independent variables, and groups were coded 0, 1, or 2 for controls, discontinuous, and continuous exposure, respectively. General impression and temperament scores were used to categorize dogs for comparison by Chi-square analysis (Table 2). Severity was not considered in the general impression and temperament scores as it is unclear how the interactions relate to severity. Dogs were given an equally weighted score if any of the possible combinations was present other than normal/normal.

Serology

All dogs were bled prior to the initial exposure, every other day for 30 days after initial exposure, then weekly for 8 months and exsanguinated prior to necropsy. At the time of collection, 0.5 mL of whole blood was inoculated into 7.0 mL of BSK II medium (22). Standard enzyme immunoassay (EIA) procedures were used to determine antibody levels to Bb proteins. Briefly, wells of a 96-well microtiter plate were coated with 200 μL coating buffer containing 1 ng of Bb protein. Proteins selected for test purposes were Osp A, B, C, and D; P39; 79.8 kD; and whole Bb strain B31 sonicate. Plates were washed, and dilutions of sera were added to the plates. Plates were again washed, and anticanine antibody conjugated to horseradish peroxidase was placed in the wells. Plates were washed a third time, and TMB substrate was added to the wells. Color development was determined using a microtiter plate spectrophotometer. Cerebrospinal fluid was collected from all dogs prior to exposure, 10 days postexposure, and at 3 and 5 months postexposure for determination of intrathecal antibody production.

Tissue sample evaluations

Dogs were euthanized, and tissues were removed, divided, and placed in 10% buffered formalin, 80% ethanol fixative, and BSK II media. The following tissues were included: brain, pituitary, spinal cord, eyes, lacrimal glands, sciatic nerve, skin, mammary, ear, muscle, diaphragm, joint capsule, liver, intestine, pancreas, tongue, esophagus, stomach, salivary gland, spleen, thymus, lymph node, bone marrow, tonsils, heart, trachea, lung, adrenals, thyroid, kidneys, reproductive tissue, and bladder. Routine histological examinations were conducted as reported in a companion paper (25). Tissues and blood samples placed in BSK II media were incubated at 31°C for up to 6 weeks. Each culture was examined weekly by dark-field microscopy, and cultures with evidence of spirochetal growth were tested by flourescene-labeled monoclonal antibody against Bb isolate B31 for confirmatory identification as Bb.

RESULTS AND DISCUSSION

Clinical observations

Evaluation of clinical signs prior to moving the dogs out of individual cages was difficult, and no observable differences were noted among the groups. Following placement in a single isolation room, mild gait abnormalities and occasional aggressive behavior were noted beginning on day 168 after initial placement of the ticks (PE) and continuing through day 188 PE. Both exposed groups showed significantly greater gait abnormalities than the nonexposed controls beginning at 189 days PE and continuing through 197 days PE (Figs. 1 and 4 through 6). One dog from the continuous group developed severe lameness on day 189 PE and was removed to a separate isolation room until day 210 PE. Physical examination revealed sensitivity when the left carpal joint was maximally flexed. The lameness resolved without further incidence or recurrence by day 197 PE. Similar signs of mild aggression and gait abnormalities were exhibited at 210 to 218, 222 to 227, 231 to 236, and 242 to 247 days PE in all groups. Overall scores indicated milder clinical signs during the subsequent cycles, and some mild gait abnormalities were noted in the nonexposed dogs (Figs. 2 and 3). A spike in gait scores in the nonexposed dogs occurred 197 to 207 days PE after which increased gait scores were recorded in this group at time in-

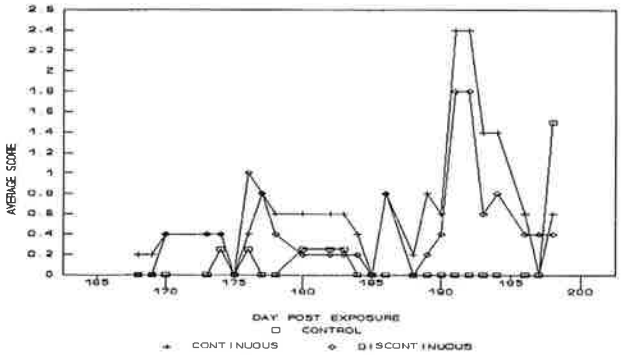

FIG. 1. Average gait score in dogs following exposure to ticks infected with Bb on days 168 to 198 postexposure.

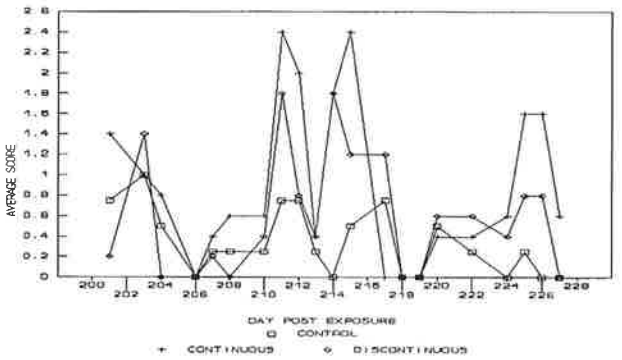

FIG. 2. Average gait score in dogs following exposure to ticks infected with Bb on days 201 to 227 postexposure.

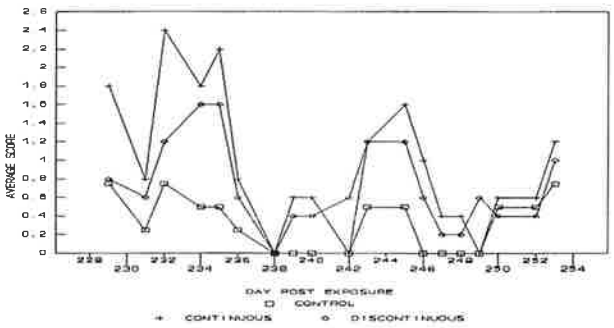

FIG. 3. Average gait score in dogs following exposure to ticks infected with Bb on days 229 to 253 postexposure.

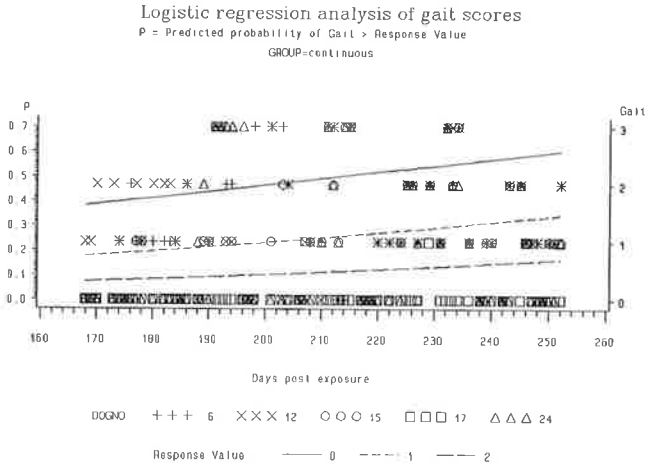

FIG. 4. Logistic regression analysis of gait scores in continuous exposure group.

tervals coinciding with the exposed dogs. Logistic regression analysis was used to assess the statistical significance of the occurrence of gait abnormalities. The periodicity mentioned earlier was not considered in this analysis. The analysis indicated a significantly higher proportion of gait abnormalities in the exposed dogs, more so in the continuous than in the discontinuous exposure group ($P \leq 0.0001$). A significant increase in the proportion of abnormal gait with time was noted ($P \leq 0.0002$). It can be determined from the data that this proportion was higher in the second half of the period of observation, not only among exposed dogs but also in the controls (Figs. 4 through 6). Therefore, no conclusion should be drawn from the time effect. Serological monitoring did not indicate exposure to the organism in the nonexposed group (24). However, because the dogs were housed together, it is possible that the organism was transmitted to these dogs. At this time, no irrefutable evidence is available to confirm or deny transmission of the organism to the nonexposed dogs. The mild clinical signs may have been elicited due to the grated flooring on which the dogs were housed. Openings in the flooring were large enough so that occasionally a dog's foot pad could become temporarily trapped between slats causing mild irritation. Physical examination indicated mild soreness in the digital pads of affected dogs. During periods of heightened aggression, the dogs became much more active. This may have increased the potential for soreness caused by the flooring, thus leading to stress-induced gait abnormalities.

Both groups of exposed dogs exhibited significantly more occurrences of general impression/temperament (GI/T) symptoms other than 0.0 (normal/normal) than the nonexposed group ($P \leq 0.05$). Because the nonexposed group remained normal/normal throughout the experiment, no further comparison was made with this group. The comparison of interest was between the dogs exposed on the continuous program and the discontinuous group, which showed a significant difference ($P \leq 0.05$). Continuous tick exposure may have increased the number of infecting organisms in these dogs. Increased numbers of organisms in the host prior to induction of a significant immune response may have affected the concentration of symptom-inducing factors, thus altering the observed response. Longer-term studies may be necessary to determine if a dose-related response is evident and if the occurrence of observed signs in dogs receiving a lower dose is time dependent.

Temperatures were unchanged in all groups throughout the course of the experiment (Fig. 7). One dog in the unexposed group exhibited a temperature that was unusually high (41°C) on days 238 and 252 PE but that was normal on the subsequent 4 days at each time period. Average baseline temperature for the groups was 39°C with fluctuations during the experiment within the normal range of ±0.5°. No correlation between temperature variations and occurrence or severity of gait abnormalities can be made from these observations.

Weight differences were noted among the groups (Fig. 8). Both groups of exposed dogs gained significantly more weight than the unexposed group, as determined by use of SAS-GLM analysis. In the analysis, time was used as an independent variable, and the interaction of time group was used to indicate

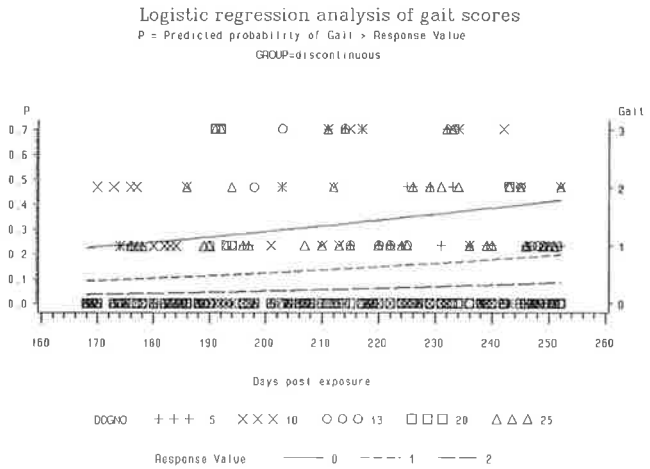

FIG. 5. Logistic regression analysis of gait scores in discontinuous exposure group.

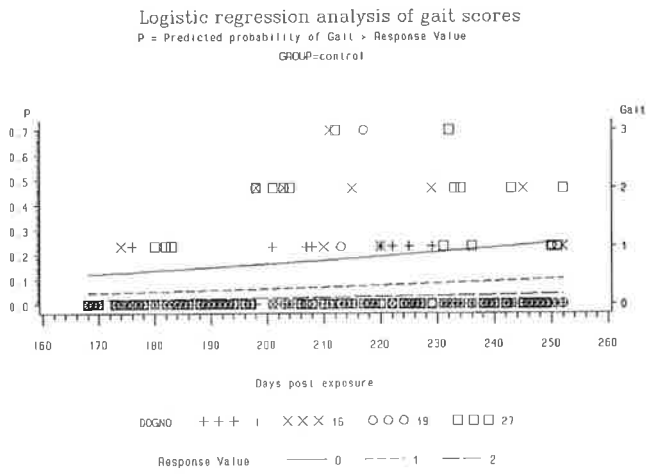

FIG. 6. Logistic regression analysis of gait scores in nonexposed group.

whether the groups had significantly different slopes. The results indicated that the common regression was significant ($P \leq 0.001$) and that the regressions of the three groups differed significantly ($P \leq 0.001$). These conclusions were reached independently from the fact that a significant difference in initial weight existed between the three groups. This finding may be due to reduced activity in the exposed groups. It is tempting to speculate that clinical signs of Lyme disease may result in reduced activity leading to increased weight gain. However, food intake was not measured, and weight gain cannot be directly linked to a specific cause.

Serology

Serological results were reported previously (24). Both P39 and Osp C were indicative of active infection while antibody levels to Osp A, B, and D, B31, and 79.8kD were inconsistent for indication of infection. Antibodies to Osp C were consistently detected at 15 days postexposure and to P39 at 30 days postexposure.

Tissue sample examinations

No significant abnormalities were noted during routine histopathological analysis, as reported in a companion paper (25).

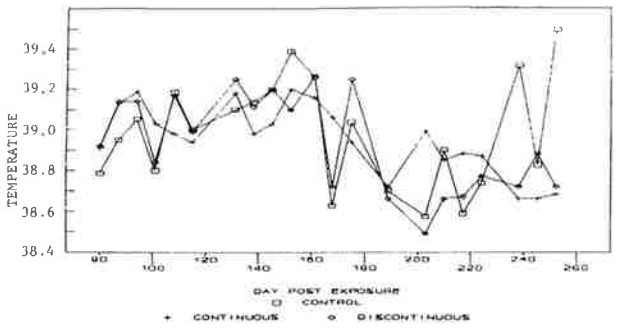

FIG. 7. Average temperature (°C) of dogs following exposure to ticks infected with Bb at weekly intervals from 80 to 252 days postexposure.

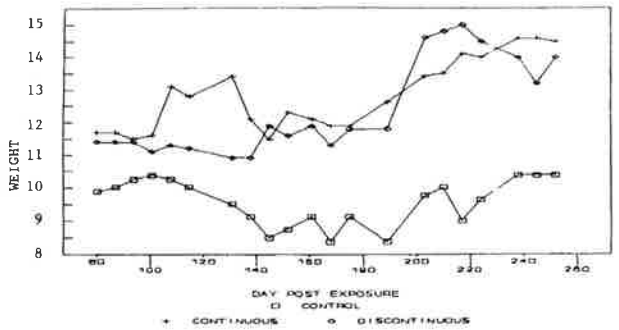

FIG. 8. Weights (Kg) of dogs following exposure to ticks infected with Bb at weekly intervals from 80 to 253 days postexposure.

Spirochetes were not recovered from tissues of nonexposed dogs. Organisms that reacted with flourescene-labeled monoclonal antibody against Bb isolate B31 were recovered from various tissues. One dog exposed in the discontinuous method yielded a positive culture from the urinary bladder. Two dogs in the continuous program had positive cultures. The synovium from one dog and the urinary bladder, meningies, and thyroid from a second dog were culture positive.

CONCLUSIONS

Clinical signs that developed in adult immunocompetent dogs following exposure to Bb-infected ticks were not as severe as those that developed following laboratory challenge previously described (16, 17). The clinical signs that were observed in this experiment correlated well with reports of normal populations of naturally exposed dogs and with reports by other researchers using a similar challenge model in younger dogs (18–21, 26).

The subtle nature of signalment observed in these dogs demonstrates the difficulty of using a specific set of clinical criteria for diagnosing subacute or chronic canine Lyme borreliosis. A future report will examine the ability of current *in vitro* diagnostic assays to follow the progression of the disease in these dogs.

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Canine Lyme Borreliosis II. Minimal Lesions in Tissues of Laboratory Beagles following Infection by Exposure to Ixodid Ticks Infected with *Borrelia Burgdorferi*

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Natural infection of dogs with *B. burgdorferi* produces lameness and both gross and histologic lesions. Attempts to model borreliosis of man and dogs by experimental infection of dogs have met with limited success. The results of such experimental infections suggest either that the disease is not well reproduced in a laboratory setting or that Lyme borreliosis in the dog is a relatively mild disease. Fourteen purpose-bred young adult Beagle dogs were experimentally infected with *B. burgdorferi* by repetitive pair feeding of infected adult ticks. Infection was confirmed by rising antibody titers following exposure and reisolation of the pathogen. The dogs were closely observed for 8 to 12 months following infection and then were killed and necropsied. An extensive panel of tissues were prepared and examined. Aside from transient lameness—localized to a single joint, in a few dogs—and altered alimentation, there were few clinical signs associated with infection. There were no infection-associated gross lesions. Vacuolation of cells of the adrenal cortex and proliferation of glomerular mesangial cells and matrix were noted in infected dogs. While it is clear that infection was established, there were few gross or microscopic lesions seen in the tissue of dogs that were caused by *B. burgdorferi*. By extrapolation from this model, Lyme borreliosis in dogs may be a relatively mild clinical disease, producing few lesions in the majority of infected dogs.

Key words: Canine, Lyme disease, *Borrelia burgdorferi*

Natural infection of dogs and other domesticated animals with *B. burgdorferi* produces a variety of clinical signs and both associated gross and histopathologic lesions. However, the incidence of both clinical disease and lesions is moderately low in animals exposed to the pathogen. Magnarelli et al. (12) found 28.6% (60/210) of dogs in southern Connecticut were seropositive, indicating exposure to *Borrelia*, and clinical lameness in 19/52 of these dogs. Antibodies to *B. burgdorferi* were also found in 67 to 77% of dogs in the lower Hudson River Valley and Connecticut. Sixty-two percent of seropositive dogs had signs of lameness, while 6% of seropositive dogs had normal gait on physical examination. Study of a comparable population of seronegative dogs from the same geographical area showed that 29% of seronegative dogs had signs of lameness, while 3% of this population was considered to be normal in gait. Other clinical abnormalities seen more commonly in seropositive dogs included elevated body temperature, fatigue, and anorexia (13).

Cohen et al. (3) found antibodies to *B. burgdorferi* in 5.5% (132/2409) of serum samples collected from dogs in Texas. Clinical records were available for 29 seropositive dogs. In these, fever was reported in 20% of seropositive dogs and lameness in 16% of them. In another study of dogs, examined in a clinical setting in borreliosis-endemic areas, Levy and coworkers (9, 10) found polyvalent antibodies to *B. burgdorferi* in 126/234 dogs. Of these seropositive dogs, 4.8% developed joint disease within 20 months; however, joint disease was seen in 4.6% of the seronegative control population they studied. In a separate clinical study, Levy et al. (11) reported clinical signs of disease in 211 dogs (out of 4498 nonvaccinated dogs) living in borreliosis-endemic areas, an incidence of 4.6%.

Joint lesions, gait abnormalities, joint pain, and arthritis are the most commonly and widely reported consequences of infection in dogs (7). In one group of 25 dogs displaying acute clinical signs (lameness, fever, lethargy, anorexia, and joint pain), 7/25 dogs had signs of pain/dysfunction in one joint, while seven other dogs had signs in two or more joints (9). In a group of 34 dogs with clinical signs, Kornblatt et al. (8) found pain/dysfunction in one joint of 14/34 dogs and pain/dysfunction in two or more joints in 20/34 dogs. A septic joint effusion was found in 9/34 dogs, but it is not clear if affected and nonaffected joints were routinely sampled. The carpus and digital joints were most commonly affected in one study (9, 10), but the hind legs were more affected in another (3).

Uniform criteria for establishing lameness (clinical examination, radiography, gait analysis, joint fluid analysis, biopsy) and exclusion of other common causes of joint dysfunction have not been consistently followed by all investigators, making it difficult to compare data from these different studies. And while a response to antibiotic therapy was suggested by some investigators as proof of borreliosis, this has not been consistently shown. Likewise, a failure to sample or to successfully recover organisms from affected joints raises some questions about the actual incidence, or importance, of *Borrelia*-associated arthritis in dogs.

Some infected dogs may develop myocarditis (9), nephritis (5, 12), neurologic dysfunction (9), or uveitis (3, 15), but the incidence of these diseases is rare, when one considers the number of exposed dogs.

Musculoskeletal problems, similar to those described, have also been observed in cattle and horses (2, 16).

There have been a number of attempts to replicate both clin-

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ical signs and tissue lesions seen in naturally occurring borreliosis of man and dogs, using experimentally infected dogs as a model. This has proved challenging.

Greene et al. (6) exposed adult Beagle dogs to *B. burgdorferi* either by a combination of tick feeding and intravenous injection or by a combination of subcutaneous and intraperitoneal injection, followed in both instances by administration of therapeutic doses of dexamethasone. They found that exposed dogs developed antibodies within 10 days of exposure and displayed an anamnestic response when challenged. However, dogs did not develop any clinical or pathological signs of disease in a 6- to 8-month observation period. The organism was not isolated from either the blood or urine of exposed dogs, despite repeated attempts.

Wasmoen et al. (19), in three separate studies, inoculated 26 mixed breed adult dogs with isolated, cultured *B. burgdorferi* by repetitive intraperitoneal/subcutaneous/intradermal injection. Following inoculation, 20 dogs were treated with therapeutic doses of dexamethasone. Twenty percent of dogs inoculated with *B. burgdorferi*, but not treated with dexamethasone, developed fever and intermittent lameness within 35 to 42 days of infection. Seventy percent of inoculated dogs treated with dexamethasone developed fever and lameness within 66 to 121 days after infection. Inappetance and anorexia was noted in some infected dogs. Spirochetemia was noted in a substantial number of dogs. The pathogen was reisolated from some dogs and then used successfully to infect other dogs, some of which subsequently developed lameness. These results were taken as proof of Koch's postulate—in this case, that infection produced arthritis and synovitis.

Appel et al. (1) exposed 56 Beagle dogs, aged 3 days to 13 weeks, and two pregnant adult females to infected nymph and adult ticks, collected from a borreliosis-endemic area. Some dogs were inoculated directly with the organism, but most were exposed by allowing ticks to feed for 4 to 6 days on shaved skin. A number of dogs were exposed several times, and at least two dogs were also given injections of dexamethasone concurrently with exposure. Acute, transient, but recurrent lameness occurred in 16 dogs, within 2 to 5 months of exposure. Adult tick bites were more efficacious than nymph bites in infecting dogs, this being a reflection of the higher rate of infection of adult ticks with *B. burgdorferi* (62 to 80% in adults versus 16% infection of nymphs). Fibrinopurulent arthritis and synovitis were seen in three of six dogs that had displayed acute lameness, when these dogs were necropsied. In one animal, *B. burgdorferi* was isolated from the joint capsule of an affected joint, but it was also isolated from a normal joint capsule of another animal that was not lame. These investigators also noted lymphoid hyperplasia in regional peripheral nodes of dogs with acute lameness and mild lymphoplasmacytic dermatitis at the site of tick bites.

We report in this and in a companion paper (4) the successful experimental induction of borreliosis in adult, immunocompetent dogs, by repeated exposure to infected adult *Ixodes dammini* ticks. Observations of dogs over a 9- to 12-month period after exposure showed behavioral changes and gait abnormalities as the major sequelae of infection.

METHODS

A detailed description of methods is given in a companion paper (4). Briefly, 14 adult (1-year-old) male and female Beagle dogs were purchased from a commercial supplier (Marshall Farms, Rochester, NY) and housed in an isolation kennel. Such commercially available dogs are considered to be an adequate model for all other breeds of dogs. Dogs were vaccinated against routine canine pathogens, fed commercial dog food, and given free access to tap water. Four dogs were randomly selected as nonexposed controls, while 10 others were exposed to infected ticks (4).

Five dogs were exposed to continuous tick feeding (a new set of tick pairs placed 1 to 2 days following detachment of the previous set), while the other five experimental dogs were exposed in a discontinuous pattern of feeding (new set of tick pairs placed 7 days after detachment of the previous set). Exposure cycles were repeated seven times for the continuous feeding group and five times for the discontinuous feeding group. Infection of dogs was confirmed by culture of ear punch biopsies (4).

Clinical signs were monitored daily and gait scores recorded. A semiquantitative scoring system for clinical observations, gait, and behavior changes was used for comparison and statistical analysis.

Serum samples were collected every other day for 30 days after exposure and weekly thereafter. Samples of cerebrospinal fluid was collected prior to and then at 10, 90, and 150 days after exposure. Antibody levels were determined by ELISA against whole-organism sonicates and 39 and 79.8 kD protein fractions.

Between 9 and 12 months after exposure, dogs were killed with an intravenous overdose of concentrated barbiturate and necropsied by a veterinary pathologist. Tissues were collected for histologic evaluation and for culture of *B. burgdorferi*. Tissues collected in 10% neutral buffered formalin included the following: brain, pituitary, spinal cord, eyes, lacrimal glands, sciatic nerve, skin and mammary gland, skeletal and diaphragmatic muscles, joint capsule, small and large intestines, liver, pancreas, tongue, esophagus and stomach, salivary gland, spleen, thymus, lymph node, bone and marrow, tonsils, heart, trachea, lung, adrenal, thyroid and parathyroid, kidney, reproductive organs, and urinary bladder.

Tissues were fixed for a minimum of 72 hours after necropsy and then dehydrated in a graded series of alcohols and xylene, embedded in paraffin, and sectioned. Tissues were routinely stained with hematoxylin eosin and periodic acid Schiff stain. All tissues were then examined by a veterinary pathologist in a blind manner, i.e., without knowledge of treatment group (control, continuous feeding, discontinuous feeding). A semiquantitative scoring system was applied to help quantify the incidence and severity of lesions seen. All lesions were recorded using a computerized data collection system (PLACES, Apoloco Systems Ltd., Newcastle, UK).

RESULTS

Clinical observations

These results are detailed in a companion paper (4).

Gross pathology

Gross lesions were seen in several tissues of both exposed and nonexposed dogs; none of the lesions were attributed to infection with *B. burgdorferi*. Scattered foci of white and red discoloration were seen in the heart and lungs of three exposed dogs. Two infected dogs had minimal enlargement of precapsular or popliteal lymph nodes, and one of these dogs also had minimal splenic enlargement. There were no microscopic lesions correlated with these gross observations. Such lesions are known to occur spontaneously in Beagle dogs of this age, maintained in these conditions of husbandry.

No gross lesions were seen in any joints examined.

Histopathology

Lesions attributable to infection were seen in the adrenal glands and in the kidneys.

The adrenal cortex in two of four female dogs exposed in a discontinuous manner showed minimal multifocal vacuolation. Cells of the zona glomerulosa and fasciculata had an increased number of minute cytoplasmic vacuoles, and such cells were also minimally enlarged.

Proliferation of the glomerular mesangium was noted in one of three male dogs continuously exposed for infection and in three of four female dogs exposed in a discontinuous manner. Affected dogs showed a minimal to moderate increase in the number of mesangial cells and amount of mesangium present. While proliferation of the glomerular mesangium can occur spontaneously in dogs, it is much more common in older (>5 years) dogs (18).

A variety of other lesions were seen in various tissues of both exposed and nonexposed control dogs. All lesions seen were of minimal significance and were of the types that occur spontaneously and commonly in Beagle dogs of this age, maintained under these conditions of husbandry.

DISCUSSION

In this study, there was little gross or microscopic pathology associated with infection by *Borrelia burgdorferi*. This is in accord with observations made in many naturally infected dogs, which have antibodies to the pathogen, but no evidence of clinical disease (3, 9–14). Likewise, previous studies of the disease in laboratory dogs also showed that the disease tends to be mild, even in dogs treated with glucocorticoids during disease induction (1, 19).

We did not find gross or microscopic lesions that correlated with lameness or behavioral changes (increased aggression, overeating), which we observed clinically. We did not anticipate seeing lesions in the brains of affected dogs, since there are rarely gross or microscopic changes that correlate with subtle behavioral changes in dogs.

We were surprised by the paucity of joint lesions. Some dogs had displayed stiffness, reluctance to walk, or frank lameness at various times after infection. These gait abnormalities were more pronounced in dogs infected by continuous exposure than in those infected by discontinuous exposure. However, these dogs had normal joints, without discoloration or erosion of joint surfaces or alterations in the capsule or synovium. Joint fluid was normal in quantity and appearance. Although lameness is reported as a common clinical sign in naturally infected dogs, there have been no studies of the joint pathology in these dogs. Without a systematic examination of the joints of a large number of dogs, it will be difficult to extrapolate from our laboratory data to the clinical setting. Several investigators (1) have noted, however, that even though *Borrelia* can be isolated for the synovium of experimentally infected animals, these dogs do not develop arthritis. It is possible that dogs may be resistant to the development of chronic degenerative joint disease as a consequence of infection. This will require further study.

Vacuolation of cells in the adrenal cortex has not been reported in other experimental studies. Appel et al. (1) noted that the adrenal glands were a preferred site for reisolation on the pathogen in their study. A direct effect of the pathogen on the adrenal remains to be proved. Likewise, it is possible that such vacuolation may reflect an increased demand for corticoids in response to the stress of infection. This, too, will require further study.

We found proliferation of glomerular mesangial cells and an increased amount of mesangial matrix in infected dogs. The mesangium plays a primary role in the clearance of antigens that lodge in capillary basement membranes. Mesangial expansion is common in dogs that are clearing immune complexes (17). It is possible that infected dogs were producing immune complexes throughout the study period and that these lodged in the kidney and were subsequently cleared, through mesangial activity. We are in the process of examining kidneys from study dogs for such complexes.

We did not see clinical signs (polydipsia, polyuria, dehydration, oral ulceration) that would indicate significant renal disease in any of our dogs. This is not surprising, as it appears that renal disease associated with *Borrelia* infection is uncommon.

We have shown that fully immunocompetent (not treated with corticosteroids) adult dogs can be infected with *Borrelia burgdorferi* through tick vectors. Dogs develop relatively mild clinical disease within months of infection. This model is very similar to the clinical course of natural infection, and the spectrum of lesions seen is also similar. We conclude that the experimentally infected dog is a suitable model for the study of naturally occurring borreliosis in dogs and can be used to develop effective therapies. However, there has been insufficient study of the pathology in either naturally or experimentally infected dogs to conclude that manifestations of infection are completely analogous to those seen in man.

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Detection of *Borrelia burgdorferi* Antigen in Urine from Patients with Lyme Borreliosis

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Lyme disease or Lyme borreliosis is a multisystem disease caused by infection with a spirochete *Borrelia burgdorferi*. In some patients diagnosed with Lyme borreliosis, the classical antibody response is slow or never develops. There are also reports of the antibody disappearing after antibiotic treatment. These reports and other enigma of Lyme disease often raise the clinical question of whether the reappearance of symptoms compatible with Lyme borreliosis after treatment are related to a reinfection or to the persistence of the original infection. The ability to observe antigenuria in Lyme borreliosis could aid in the clinical assessment and management of these difficult patients. This paper presents the development of an antigen assay for *B. burgdorferi* based upon detecting the presence of *Borrelia* antigen in the urine of patients with Lyme borreliosis and discusses the relationship of the antibody response to the presence of antigen. An antigen "capture" competitive inhibition assay was developed that can detect *B. burgdorferi* antigen in the urine of patients. Antigen was typically detected early in the course of disease, but it was also seen in some patients a year or more after the erythema migrans (EM) rash. In this method, antigen was captured by a unique polyclonal antibody before it could compete with antigen bound in the solid phase. The antibody used was a specifically absorbed polyclonal antibody, which had reactivity only against the 31, 34, 39, and 93 kDa antigens of *B. burgdorferi*. The affinity of the antibody and the nature of the assay allowed specific detection of low levels of antigen, in spite of the presence of other proteins. Serum and urine samples were obtained from more than 700 patients (425) and normal controls. After single-blind laboratory analysis, the results were correlated with clinical examination results and patient history. It was found that 30% of patients with Lyme disease (251 EM positive) had a positive Lyme Urine Antigen Test (LUAT) and 8% had a concurrent positive serology. The LUAT was positive in all three phases of disease: early (less than 60 days), before serology was positive; during treatment (60 days to 1 year); and a late period (greater than 360 days), when serology was often negative. Although Lyme borreliosis is defined by clinical diagnosis, various markers from the laboratory, such as specific antibodies and antigen, can aid in this process. The presence of specific antigen of *B. burgdorferi* in the urine of patients with Lyme borreliosis may be an adjunctive marker to the clinical diagnosis and traditional serological assays.

Key words: Lyme Disease, LUAT, Lyme Antigen Test, In-vitro assay, Antigen Capture, Urine

Lyme borreliosis is caused by an infection with the spirochete *Borrelia burgdorferi*, normally transmitted via a tick bite. This disease is reportedly the leading arthropod-borne illness in the United States and causes disease in Asia and Europe (1, 2).

Although Lyme borreliosis can respond favorably with early antibiotic treatment (3), the disease may be missed during its early stages (4). While erythema migrans (EM) is the characteristic diagnostic rash, it does not appear in all patients. Even in those patients in which EM appears, it may be overlooked or misdiagnosed as another type of insect bite. Clinical signs of the disease are frequently attributed to other causes. Symptoms can include a flulike illness, headache, fatigue, muscle or abdominal pain, cardiac and neurological abnormalities, and arthritis (3, 5).

Current laboratory tests for Lyme borreliosis are serological assays to evaluate IgG and/or IgM specific antibodies to *B. burgdorferi* (4, 6, 7) and include IFA, enzyme-linked immunosorbent assay (ELISA), and Western Blot. However, there is no universally defined reference standard, and patients may test negative on one assay and positive on another due to immune response variability and the complex nature of the *B. burgdorferi* antigens (8–10). This could explain in part the reports of variability in results between laboratories (11). In addition, all serum antibody tests suffer from a higher false-negative rate in the early stages of the disease because antibodies may not be produced in detectable quantity until several weeks after infection (3, 6). It has also been suggested that early but inadequate antibiotic treatment may prevent full antibody development in clinically positive patients, mask clinical symptoms, and not completely eradicate the organism (6).

Testing accuracy or the ability to have greater sensitivity without sacrificing specificity increases in the later stages, but false-positives are still known to occur due to cross reactivity with syphilis, mononucleosis, some autoimmune diseases, and possibly periodontal disease (3, 9, 12). In addition, there is a report that in some cases, immune complexes may mask the serological response (13). Therefore, most clinicians recommend that a diagnosis of Lyme borreliosis be based on clinical signs and symptoms, with multiple laboratory tests being used only as supportive data (5, 8, 14). Assays that focus on the detection of some of the more unique antigens of *B. burgdorferi* may help provide additional laboratory tools to aid in the diagnosis of Lyme borreliosis.

In 1989, Hyde et al. (15), using multiple monoclonal antibodies in a dot blot assay, reported the detection of specific *B. burgdorferi* antigens (31, 34, and 41 kDa) in the urine of mice and humans. Coyle et al. (16) detected antigen in the cerebrospinal fluid (CSF) of neurological patients with presumed *B. burgdorferi* infection using monoclonal antibodies in an antigen-capture ELISA. Dorward et al. (17) used a rabbit polyclonal antibody in an electron microscopic, immune capture assay, and detected antigen of *B. burgdorferi* in the urine of mice and humans. Unique to this study was the observation that small fragments of *Borrelia* antigen, rather than whole organisms, were the more likely finding. Reports (18) have also indicated the detection of spirochetal DNA in the urine

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of patients with Lyme borreliosis. It was not clear from any of the above mentioned studies whether the bladder or the urinary tract itself was a unique site for the spirochete. A study of Magnarelli et al. (19) in mice detected infected bladders in 95% of mice with antigen in the urine.

The objective of the current report was to measure antigen in the urine and antibodies in the serum of patients with Lyme borreliosis. The Lyme Urine Antigen Assay (LUAT), thus developed, was a special antigen capture-inhibition ELISA with a unique absorbed polyclonal antibody with binding activity to 31, 34, 39, and 93 kDa antigenic moieties.

MATERIALS AND METHODS

Study design

The initial studies of the LUAT were designed to examine negative control groups as well as patients suspected of having Lyme borreliosis. The LUAT was performed single-blind on more than 700 patients and negative controls.

Serum and urine specimens from patients presenting with symptoms of Lyme borreliosis (n = 425) were submitted in a single-blind fashion to the laboratory. The minimal criteria (20) for inclusion in this group were tick bite, being from an endemic area, and three or more recognized symptoms of Lyme disease. Samples were sent frozen and kept at −20°C until analyzed. After analysis, a clinical study monitor assembled the data from a uniform history form that had recorded the data of a physician observed EM, other laboratory data, history, and current signs and symptoms. In addition, previous and current antibiotic treatment were noted.

After the initial data analysis, the clinical study monitor established a subgroup of patients (n = 251) meeting the tighter CDC surveillance case definition (21). These patients all came from a recognized endemic area of New Jersey, Connecticut, or New York; all had a physician-diagnosed EM; and all had three or more of the recognized clinical manifestations of Lyme borreliosis. Specimens were obtained from patients in all three phases of clinically diagnosed Lyme disease. The phases were defined as early, within 60 days of the EM; medium, between 60 days and 1 year of the EM; and late, more than 1 year after an EM.

The first normal control group (n = 208) was made up of individuals in an endemic area (Minnesota and Wisconsin, n = 139) and a nonendemic area (California, n = 69) with no symptoms or history of Lyme borreliosis or syphilis. A second control group (n = 50) came from the endemic area of New York and New Jersey. In addition, a third, special urine control group of patients with arthritic symptoms (n = 150) was established. All the patients, in the third control group, had either arthritis or arthralgias but no history or evidence of Lyme borreliosis, syphilis, systemic lupus (SLE), or scleroderma. This last group of patients came from all over the United States, with no geographic predominance.

Serological ELISA and antigen-capture ELISA

The FASTLyme serology assay was performed as previously reported (22). The overall format of the LUAT assay is presented in Fig. 1. In the LUAT, antigen in urine competes with antigen bound on the solid phase. Captured antigen in the urine blocks the binding of the antibody to the solid phase and inhibits the development of fluorescence in the ELISA assay (23).

Negative controls were prepared from normal urine samples from healthy employees with no symptoms or indications of Lyme disease. Positive controls were prepared from assay positive urine samples from patients with clinically diagnosed Lyme borreliosis. Calibrators were made by spiking urine with sonicated B31 antigen at various concentrations. The 400 ng/mL calibrator was also used as one of the positive controls.

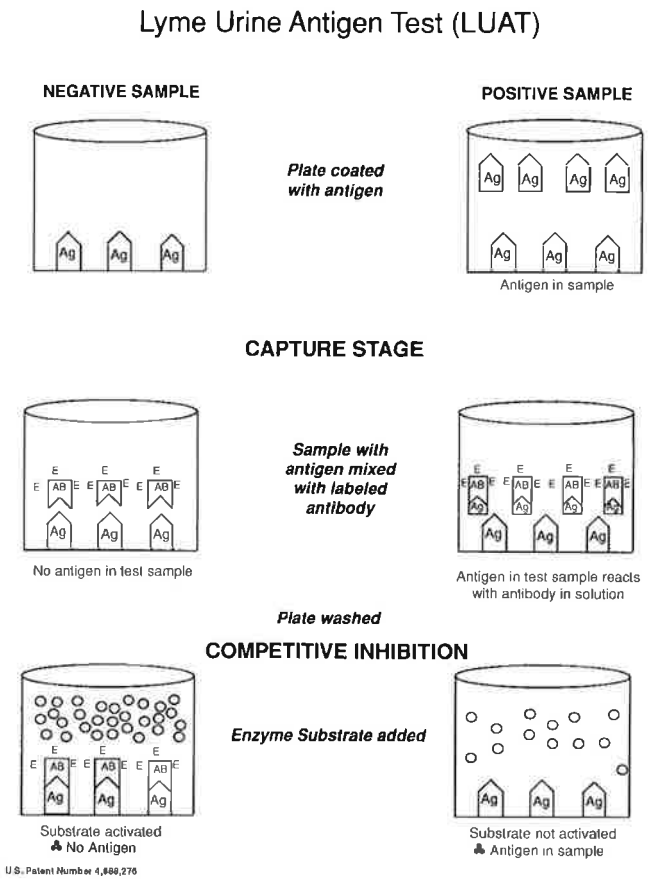

FIG. 1 Design of the LUAT.

The LUAT assay was run in duplicate. The within-run coefficient of variation (CV) was less than 10% and the run-to-run CV was less than 15%. One milliliter of thawed patient or control urines (ph 5 to 7) were spun at 12,000 × G for 10 minutes. Previous analysis showed that specific antigen of *B. burgdorferi* was found in both the pellet and supernatant but more consistently found in the pellet. Therefore, the supernatant was discarded. Since the pellet is used, urine specimens with gross cellular and gross bacterial contamination were excluded from the study. (We theorize that gross contamination may cause actual physical interference in the washing steps. From the blocking and spiking studies a reasonable amount of contamination has no effect on the assay system.) The pellet was resolubilized with 400 μL of 0.09 M Tris buffer at a ph of 7.4. This solution (ph 7.4 to 7.6) was then incubated with *B. burgdorferi* specific polyclonal antibody, conjugated with alkaline phosphatase for 1 hour at 37°C. Controls and calibrators were processed similarly.

The antibody used was from a unique pool of three rabbits hyperimmunized with sonicated, low passage, strain B31 of *B. burgdorferi*. To obtain the sonicate, 10 mL of a culture of *B. burgdorferi* were chilled in an ice bath and sonicated with a Tekmar sonicator (Cincinnati, OH), Tip Mod. No. CV 17 at a duty cycle of 60% and a tip limit of 3. Duration was for 1 minute, then pause for 1 minute. This cycle was continued

until no whole spirochetes could be observed under the microscope with 100× oil. The sonicated material was initially passed through a 0.8-μm filter and then a 0.22-μm filter. The rabbits were each initially injected with 500 mcg of sonicated *B. burgdorferi* antigen with Freund's complete adjuvant. They were boosted every 3 weeks with 100 mcg of antigen in Freund's incomplete adjuvant and test-bled until they had the appropriate response. This antibody was chosen because its reactivity to positive control samples most closely resembled three monoclonal antibodies developed by 3M Corporation (St. Paul, MN) against the OSP A (31 kDa), Osp B (34 kDa), and flagella (41 kDa) proteins and used in the initial published studies of a urine antigen test for *B. burgdorferi* (15). After absorption with common bacteria from both normal urines and from some patients with urinary tract infections (UTIs), Western blot analysis (Fig. 2) of one of the strips cut from a run of negative and positive controls, patients and molecular weight markers demonstrated antibody activity only against 31, 34, 39, and 93 kDa antigenic moieties. The reactivity against 31 and 34 kDa appeared identical to that seen with the monoclonal antibodies (31 and 34 kDa) previously studied (15), and the reactivity to 39 kDa was distinct and different from that seen with the monoclonal antibody to 41 kDa.

Microtitration wells (MicroFluor B, Dynatech Corp., Chantilly, VA) were precoated with the sonicated, low passage, B-31 strain of *B. burgdorferi*. Wells were prewashed, and the test solution was added. Plates were incubated for 2 hours at controlled room temperature (21 to 23°C). The plates were then washed three times with the Tris buffered saline, ph 7.4, containing Triton X-405 detergent used to wash the pellet, and 100 μL of fluorescence substrate (4-methyl umbelliferyl phosphate) was added. The plate was then incubated for 20 to 30 minutes at controlled room temperature and read in a Dynatech fluorometer at 450 nm.

RESULTS

Blocking and interference studies

Blocking and interference studies were performed as follows: (1) negative patient urines were spiked with various concentrations of human serum protein; (2) negative patient urines were spiked with multiple concentrations of either whole blood, serum, RBCs, or WBCs; (3) antigen positive urines were spiked with either whole blood, serum, RBCs, or WBCs. Negative urines spiked as in action (1) or (2) above, remained negative, and no false-positives were detected. Antigen positive urines at values of 50 and 100 ng/mL retained 95 to 105% of their value when spiked with either HSA, blood, or blood components.

Normal control groups

An initial control group (n = 208) of individuals, characterized as negative for Lyme borreliosis by history and symptoms, was tested for the presence of antigen by the LUAT. These controls came from both an endemic area (Minnesota and Wisconsin, n = 139) and a nonendemic area (California, n = 69). This first control group had a 3% false-positive rate. Those seven control individuals who tested positive were lost to additional clinical follow-up.

A second control group (n = 50), more highly qualified than the first, was obtained (New York Biologics, Inc., New York, NY) from an endemic area of New York and New Jersey. All individuals in this group tested negative by the LUAT for the presence of any Lyme antigen.

Because of the high incidence of Lyme borreliosis patients appearing to have arthritic symptoms (Table 1), an additional control group was established. In this third study, urine from 150 patients from all over the United States, with arthritis and arthralgias, was examined. Patients were excluded from this study if they had Lyme borreliosis, syphilis, SLE, or scleroderma. Only one arthritic control exhibited a positive antigen value. Upon further study, this individual was found to have a UTI. This is the only listed contraindication to LUAT testing, because of the physical interference a large number of bacteria sometimes have with the ELISA format of the LUAT assay. (In the normal clinical practice of the laboratory all positive

FIG. 2 Western blot of the absorbed polyclonal antibody used in the LUAT.

TABLE 1
Results of LUAT Testing in Total Population of Lyme Disease Patients (n = 425)

Characteristic	Number (*n*)	Percentage (%)
Physician-diagnosed EM	251	59
History of tick bite	210	49
History of both EM and tick bite	133	31
>3 other symptoms	380	89
History of arthritic symptoms	306	72
Positive concurrent serology	32	8
Positive Lyme Urine Antigen Test	124	29
Antibiotic treatment	261	61

TABLE 2
Results of LUAT Testing in Lyme Disease Patients with a Physician-Diagnosed EM (n = 251)

Characteristic	Number (*n*)	Percentage (%)
History of tick bite	133	53
>3 other symptoms	204	81
History of arthritic symptoms	177	71
Positive concurrent serology	19	8
Positive Lyme Urine Antigen Test	75	30
Antibiotic treatment	159	63

LUAT patients are tested by Multistix, Miles Inc. and any suspicion of UTI is reported back to the physician.)

Lyme borreliosis patients

Patient samples from endemic areas of New York, New Jersey, and Connecticut were submitted to the laboratory. The samples were run single-blind by the laboratory. History and clinical information were stored and analyzed separately by the clinical study monitor. Of the patient samples submitted, only 425 patients met the criteria of a presumptive diagnosis of Lyme borreliosis and had a clinical history of either a physician-diagnosed EM or a tick bite with at least three major symptoms of Lyme borreliosis. The symptoms considered for the acute phase were "flulike" (which included fatigue, fever, headache, mild stiff neck, arthralgia, and/or myalgia). The symptoms considered for the later manifestations include any of the following *when an alternate explanation is not found* (20, 21): involvement of the musculoskeletal system, including arthritis in one or more joints; involvement of the nervous system, including lymphocytic meningitis, facial palsy, and radiculoneuropathy; and involvement of the cardiovascular system, including acute onset atrioventricular conduction defects. In all cases, concurrent serum and urine tests were performed on the samples.

The total data from this group of patients with Lyme borreliosis is presented in Table 1. Table 2 is a subgrouping of Table 1 and considers *only* those patients with a physician-diagnosed EM. The patients in Table 2 seem to meet the more stringent CDC Lyme borreliosis national surveillance case definition (21). It appears by an analysis of the data in both Tables 1 and 2 that the LUAT is positive in 30% of the patients. From a review of the patients' clinical history, more than 40% of the patients did have a positive serological response sometime in the course of their disease. However, only 8% of the patients in this current study were concurrently seropositive and antigen positive. The most common clinical finding in these Lyme borreliosis patients was the presence of arthritic symptoms.

TABLE 3
Analysis by Phases of Disease of Lyme Patients with EM and Positive LUAT (n = 75)[a]

Early (<60 days)		
>3 other symptoms	9/18	50%
Previous or current positive serology	2/18	11%
Medium (60 days to 1 year)		
>3 other symptoms	21/24	88%
Previous or current positive serology	7/24	29%
Late (>1 year)		
>3 other symptoms	27/29	93%
Previous or current positive serology	7/29	24%
Unknown		
>3 other symptoms	4/4	100%
Previous or current positive serology	1/4	25%

[a]Stage of disease is defined from the time of the EM.

Table 3 is an analysis, by phase of disease, of the patients with a physician-diagnosed EM who also had a positive LUAT. The arbitrary phases—early, middle, and late—were determined from the date of the EM defined by the diagnosing physician. From the analysis of these data, it appears that antigen can be detected both early and late in the disease process.

DISCUSSION

These studies demonstrate that antigen of *B. burgdorferi* can be detected in the urine of a significant number of patients with Lyme borreliosis. Furthermore, there is a significant difference ($p > 0.001$) between the expression of this antigen in the urine of normal individuals from endemic and nonendemic areas as compared with patients with clinically diagnosed Lyme borreliosis.

There was no significant difference between control groups from endemic and nonendemic areas. Since arthritic symptoms were such prominent characteristics of patients with Lyme borreliosis, a comparison was made between non-Lyme patients with arthritic symptoms and the endemic and nonendemic normal controls. Again, there was no difference with respect to antigen detection between the normal controls and the patients with arthritis and arthralgias.

Those controls with the arthritic symptoms had less than a 1% false-positive rate, but that could reflect tighter entrance criteria used for this control study. There have been reports (24, 25) that some patients diagnosed with SLE or scleroderma may have DNA of *B. burgdorferi* in their blood or urine. The first control group studied did not exclude individuals with these diseases, but none were known to have been included. The arthritic symptom control study had specific exclusions for these conditions.

In the patient groups, a history of EM or tick bite with a combination of clinical symptoms were most effective in confirming Lyme borreliosis. However, the LUAT identified three to four times as many patients (124 versus 29) with Lyme borreliosis as the concurrent serology test (Table 1), possibly due to the preselection of patients, as previously described. Among patients with a history of EM (CDC surveillance criteria—Table 2), the LUAT identified 30%, while the antibody test identified only 8%.

Table 3 reviewed only the patients with EM and a positive LUAT, divided into phases of disease based on the initial appearance of the EM. This analysis suggests that antigen in urine is present at various times during all three phases of

disease. The LUAT may be a useful diagnostic tool not only early in the disease process prior to the development of a serological response but also late in the disease process when the serological response has disappeared.

Some recent presentations (26, 27) have suggested the transient nature of antigen in urine. In those reports, antigen was present but not on a daily basis. This may be the explanation for the observation that the LUAT is sometimes negative in the face of an active infection. It was not clear from those studies whether the variation seen was due to assay performance or patient physiology. By use of the LUAT, those questions could be resolved because the LUAT is a highly controlled and reproducible assay. Future studies need to follow patients either daily or every other day after infection to monitor antigenuria. In addition, a weekly monitoring of serological response would be helpful.

There now exist a number of different tests for both antigen and antibody detection (12) for use with the clinical diagnosis of Lyme borreliosis. It is important to perform panels of tests in both serum and urine. This practice is done in other diseases, such as hepatitis, where multiple tests for both IgM and IgG antibody as well as tests for various types of antigens are routinely performed.

CONCLUSION

Lyme borreliosis is an increasingly common disease that is often difficult to accurately diagnose using only clinical symptoms. Without a physician-confirmed EM, it is particularly difficult to diagnose Lyme disease early in the disease process when treatment is most effective and long-term sequelae may be prevented. Recurrence of "Lyme-like" symptoms after antibiotic treatment is often a diagnostic dilemma. LUAT may prove to be a useful addition to current serological laboratory tests in assisting the differential diagnosis of Lyme borreliosis from other conditions presenting with similar symptoms. No single assay can work in all phases of diagnosis. Multiple laboratory tests should be used, with the clinical evaluation, to help in the diagnosis.

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The above studies were originally approved by the IRB at 3M Corp., St. Paul, Minnesota.

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Unilateral Facial Paralysis Associated with Borreliacidal Activity against *Borrelia Burgdorferi* Sensu Stricto C-1-11

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In the summer of 1993, a farm dog that had never been out of Missouri developed an acute right unilateral facial paralysis (Figs. 1 through 4). On veterinary examination, there were no other significant findings such as fever, otitis, or lameness. There was no prior history of head trauma, otitis media, other distinct illness, or Lyme vaccination. The dog, in addition to being allowed outside on the farm, was also in the owners house on a daily basis and was therefore monitored closely. Ticks were removed from the dog on a regular basis. The Bell's palsy showed dramatic improvement after therapy with tetracycline 250 mg q.i.d. × 14 days and prednisone 5 mg q.d. × 5 days. After the antibiotic therapy and resolution of the unilateral facial paralysis (Fig. 5), the owners also reported the 8-year-old, 48-pound dog was more playful, energetic, etc. and appeared much healthier. There has been no relapse of symptoms since therapy. Significantly, the farm homesite and the adjacent farm were tick collection sites for a previous Lyme survey where *Dermacentor variabilis* and *Amblyomma americanum* ticks infected with spirochetes reactive to *Borrelia burgdorferi*–specific monoclonal antibody H5332 were detected (1). Additionally, *B. burgdorferi* isolates from the same county have been cultured from *Ixodes dentatus* ticks (2). This case may have added importance since dogs were recently shown to be competent reservoirs for *B. burgdorferi* (3).

A Lyme IFA was performed at the Texas Department of Health and was borderline at 1:128. However, the dog serum was nonreactive when tested for borreliacidal activity against *B. burgdorferi* sensu stricto isolate 297. In contrast, significant borreliacidal activity was observed in both serum samples tested against *B. burgdorferi* sensu stricto C-1-11, an isolate representative of an additional U.S. seroprotective group. The C-1-11 *B. burgdorferi* isolate was originally found in *M. pennsylvanicus* voles captured in northeastern Illinois (4).

The SDS-PAGE analysis and specific reaction with monoclonal antibodies H5332 (Osp A) and H9724 (flagellin) demonstrate C-1-11 relatedness with other *B. burgdorferi* isolates. Additionally, Osp A gene sequencing indicates approximately 80% DNA homology with B31. The C-1-11 *B. burgdorferi* isolate does induce arthritis in the hamster model (5). Research has shown that *B. burgdorferi* isolate C-1-11 in the borreliacidal assay was killed only by homologous serum and represents an additional seroprotective group present among North American isolates (6).

Although unilateral facial paralysis is a common manifestation of Lyme borreliosis in humans (7), it is not a typical presentation in dogs (8). It is also known that in humans certain strains of *B. burgdorferi* can be more likely than others to cause certain symptoms—e.g., skin versus arthritic versus neurological (9). Since this case was recently presented at the VI International Conference on Lyme Borreliosis (10), continued follow-up for nearly two years since the facial paralysis has shown that the dog has remained asymptomatic. Further work is necessary; however, these results suggest this animal may have had Lyme disease resulting in a unilateral facial paralysis caused by spirochetes from a recently described molecularly distinct *B. burgdorferi* sensu stricto group found in the United States.

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FIG. 1 Acute right unilateral facial paralysis.

FIG. 2 Acute right unilateral facial paralysis.

FIG. 3 Acute right unilateral facial paralysis.

FIG. 4 Acute right unilateral facial paralysis.

FIG. 5 Complete recovery after antibiotic therapy. Two positive Borreliacidal antibody test to C-1-11 *Borrelia burgdorferi* isolate only.