Detection of *Borrelia burgdorferi* DNA in Museum Specimens of *Peromyscus leucopus*

William F. Marshall III, Sam R. Telford III, Paul N. Rys, Barbara J. Rutledge, Dane Mathiesen, Stephen E. Malawista, Andrew Spielman, and David H. Persing

Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts; Division of Experimental Pathology and the Molecular Microbiology Laboratory, Department of Laboratory Medicine and Pathology, and Division of Infectious Diseases, Mayo Clinic, Rochester, Minnesota; Section of Rheumatology, Department of Medicine, Yale University School of Medicine, New Haven, Connecticut

To determine whether *Borrelia burgdorferi* was enzootic within the United States at the beginning of the 20th century, ear skin samples taken from museum specimens of the white-footed mouse (*Peromyscus leucopus*) were examined for evidence of spirochetal DNA. In total, 280 samples from mice collected between 1870 and 1919 were analyzed by a nested polymerase chain reaction protocol. Of these, 2 specimens from the vicinity of Dennis, Massachusetts, during 1894 were reproducibly positive for *B. burgdorferi* OspA sequences. The remaining 278, representing both currently endemic and nonendemic sites, were negative for spirochetal DNA. These studies suggest that the agent of Lyme disease was present in a suitable reservoir host in the United States before the turn of the century and provide evidence against a hypothesis of recent introduction of this zoonotic agent to North America.

Although human clinical cases presenting with musculoskeletal, dermatologic, and neurologic manifestations consistent with Lyme disease were reported in Europe around the turn of the century [1], the nosologic recognition of this zoonosis did not occur until the mid-1970s, when an emerging epidemic was described in residents of coastal New England [2]. The paucity of reports of erythema migrans, the pathognomonic skin lesion of early Lyme disease, within the American medical literature before 1969 has been interpreted as evidence for a recent introduction of the spirochetal agent, *Borrelia burgdorferi*, to this continent. Such a hypothesis has been supported by observations of the limited genetic heterogeneity of American *B. burgdorferi* isolates compared with those from Eurasia. However, the presence of spirochetal DNA within *Ixodes dammini* specimens dating to the 1940s established that the agent of Lyme disease had been enzootic in the United States at least a generation before the first reported American Lyme disease cases [3]. In that study, we were unable to locate tick specimens from likely sites of spirochetal transmission that were collected before the 1940s. To further examine the hypothesis that the agent of Lyme disease had been introduced to North America during this century, we determined whether spirochetal DNA sequences were present within ear samples taken from museum study skins of rodent reservoirs (primarily *Peromyscus leucopus*, the white-footed mouse) that had been collected during the late 1800s.

Received 7 February 1994; revised 29 April 1994.

Presented in part: Fifth International Conference on Lyme Borreliosis, 30 May–2 June 1992, Arlington, Virginia.

Grant support: National Institutes of Health (AI-19693 to S.R.T. and A.S.; AI-30358, AR-41497, and AI-32403 to D.H.P.).

Reprints or correspondence: Dr. David H. Persing, Divisions of Experimental Pathology & Clinical Microbiology, Dept. of Laboratory Medicine & Pathology, Mayo Clinic/Mayo Foundation, Rochester, MN 55905; or Dr. Sam R. Telford III, Dept. of Tropical Public Health, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115.

The Journal of Infectious Diseases 1994;170:1027–32
© 1994 by The University of Chicago. All rights reserved.
0022-1899/94/7004-0045$01.00

Materials and Methods

Specimens and sample preparation. Punch biopsy specimens (2 mm diameter) were obtained from the pinnae of mouse study skins for polymerase chain reaction (PCR) testing. Such specimens consisted of intact skins that were dusted with a 3:2:1 (vol/vol/vol) mixture of borax, alum, and arsenic, respectively, then stuffed with cotton and dried. In an initial experiment, we similarly treated ear samples from 5 xenodiagnosis-confirmed infected *P. leucopus* trapped during 1990 on Nantucket Island and from 5 noninfected mice from our laboratory colony to determine whether the preservation process reduced the sensitivity of the PCR assay. Subsequently, 280 ear punch samples were removed from the rodent specimens stored at the Harvard Museum of Comparative Zoology (MCZ; Cambridge, MA), the Smithsonian Institution (SI; Washington, DC), and the American Museum of Natural History (AMNH; New York). Original dates of collection ranged from 1870 to 1938 (table 1). All samples were processed by using a clean, flame-sterilized punch and stored in separate, sterile plastic 0.5-mL microcentrifuge tubes. Most skin specimens were washed in 1.0 mL of sterile water to remove borax and arsenic compounds. The rehydrated samples were then digested with proteinase K in a 50-μL volume of K buffer (50 m*M* TRIS, pH 8.3, 1.5 m*M* $MgCl_2$, 0.45% Nonidet P-40, 0.45% Tween 20, 10 μg/mL proteinase K [IBI, New Haven, CT]). Samples were digested for 1–48 h at 55°C, depending on a visual assessment of the completeness of proteinase diges-

Downloaded from http://jid.oxfordjournals.org/ at Penn State University (Paterno Lib) on September 15, 2016

Table 1. Specimens tested for the presence of *B. burgdorferi* DNA.

Museum, rodent species	Location	Date collected	No. tested
Museum of Comparative Zoology			
PLN	Hudson, MA	February 1870	1
PLN	Hudson, MA	May 1870	2
PLN	North Carolina	December 1891	1
PLN	Liberty Hill, CT	November 1892	1
PLN	Cambridge, MA	December 1892	3
PLN	Monomoy, MA	March 1893	1
PLN	Muskeget, MA	June 1893	2
PLN	West Tisbury, MA	June 1893	1
PLN	Wareham, MA	July 1893	2
PLN	Hartford, CT	November 1893	2
PLN	Monomoy, MA	December 1893	2
PLN	South Dennis, MA	May 1894	4
PLN	South Wellfleet, MA	May 1894	2
PLN	West Dennis, MA	June 1894	1
PLN	West Virginia	April 1895	1
PLN	Barnstable, MA	May 1898	2
PLN	West Tisbury, MA	June 1899	1
PLN	Nantucket Island, MA	July 1899	3
PLN	Muskeget, MA	July 1899	1
PLN	Block Island, RI	Aug 1899	2
PLN	Ft. Adams, RI	July 1900	1
PLN	Sandy Neck, MA	December 1904	2
PLN	Naushon Island, MA	April 1905	1
PLN	Edgartown, MA	July 1910	2
Total			41
Smithsonian Institution			
PLN	Tuckerton, NJ	June–July 1870	5
PLN	Montauk, NY	September 1893	15
PLN	Shelter Island, NY	October 1894	2
CG	Two Harbors, MN	May 1895	16
PLN	Atlantic County, NJ	March 1896	5
PLN	Lake Grove, NY	April 1896	2
PLN	Sandy Hook, NJ	May 1902	2
PLN	Cos Cob, CT	1908–1909	2
PMA	Emigrant Gulch, MT	1908	15
PMA	Stevensville, MT	1908	5
CG	Danbury, WI	August 1918	3
CG	Longlake, WI	August 1918	1
PLN	Longlake, WI	August 1918	7
CG	Namekagon Lake, WI	May 1919	5
PMG	Oriental, WI	June 1919	5
PMG	Phillips, WI	August 1919	5
PMG	Solon Springs, WI	August 1919	2
PLN	Wheeler, WI	August 1919	1
PLN	Withee, WI	August 1919	1
PMG	Herbster, WI	August 1922	5
PLN	Warden, WI	July 1925	4
Total			108
American Museum of Natural History			
MP	Naushon Island, MA	September 1863	1
PLN	Ft. Snelling, MN	October 1870	18
PLN	Miller Place, NY	December 1887	4
MP	Patchogue, NY	August 1888	1
PLN	Erie, PA	September 1889	2
PLN	Erie, PA	September 1889	2
PLN	Hastings, NY	~1890	1

Downloaded from http://jid.oxfordjournals.org/ at Penn State University (Paterno Lib) on September 15, 2016

Table 1. (*Continued*)

Museum, rodent species	Location	Date collected	No. tested
PLN	Ft. Snelling, MN	October–November 1890	12
PLN	Ft. Snelling, MN	October 1890	9
PMB	Ft. Snelling, MN	October 1890	20
PLN	Hastings-on-Hudson, NY	February 1891	4
PLN	Shelter Island, NY	October 1893	1
PLN	Fairview, NJ	October 1893	9
PLN	Gardiner's Island, NY	July 1902	1
PLN	Amagansett, NY	March 1907	1
PLN	Fire Island, NY	November 1911	2
PLN	Mastic, NY	November 1913	2
PLN	Montauk, NY	August 1913	1
PLN	Mastic, NY	November 1913	1
PLN	Fisher's Island, NY	September 1924	2
TS	Lyme, CT	July 1926	3
PLN	Fisher's Island, NY	January 1926	1
PLN	Clinton, CT	August 1928	9
PLN	Dewhurst Township, WI	July 1928	5
PLN	Clark County, MN	July 1928	3
PLN	Chatham, MA	1934	6
PLN	Waterford, CT	August 1938	10
Total			131

NOTE. PLN, *Peromyscus leucopus noveboracensis;* CG, *Clethrionomys gapperi;* PMB, *Peromyscus maniculatus bairdii;* PMG, *Peromyscus maniculatus gracilis;* MP, *Microtus pennsylvaticus;* TM, *Tamias striatus.*

tion and dissolution of particulate matter. Proteinase K was inactivated by boiling for 10 min, and digested solutions were analyzed immediately or stored at −20°C in a building different from where PCR amplification was done. Strict observation of PCR product contamination control practices was observed (see below).

Detection of B. burgdorferi DNA. A 156-bp portion of the *B. burgdorferi ospA* gene was targeted for amplification by using nested primers in a PCR protocol that was recently described. Briefly, 5-μL portions of the processed specimen were used for amplification by PCR. Nested amplification was used to aid in the amplification of damaged DNA that might have been present after long-term storage under oxidative conditions. The initial round of amplification was done for 30 cycles; a 5-μL aliquot of this mixture was then transferred to a second reaction mixture containing the internal primers. Another 30 cycles of amplification followed. Products were then resolved by electrophoresis in a 1% Seakem, 3% Nusieve agarose gel (FMC Bioproducts, Rockland, ME) and visualized with UV light after staining with ethidium bromide. DNA was then transferred to a nylon membrane and hybridized with a ^{32}P-labeled internal probe as described [3]. A single positive and multiple negative controls were included in each PCR run to ensure reagent purity.

Because of the risk of contamination and resulting false-positive PCR results, we took several steps to ensure the accuracy of our results. First, the pre-PCR preparation of reagents occurred in a dedicated facility located on a different floor in a building separate from where the post-PCR analysis laboratory was located. No amplified products and no *B. burgdorferi* DNA was ever extracted, manipulated, or analyzed in this room. In addition, in the first amplification reaction of the nested procedure, dUTP was used in place of TTP in the reaction mixture, thus allowing the use of uracil DNA glycosylase to selectively degrade previously amplified DNA in the event of contamination by a previously amplified product [4]. In the second (nested) PCR reaction mixture, isopsoralen treatment was used, which is effective in eliminating up to 10^{10} copies of the first round and final reaction products [4]. Before the reaction tubes were opened, they were irradiated by UV light, rendering their contents inactive as templates for PCR [4].

Sequence analysis of the recovered fragment. The 156-bp *B. burgdorferi*–specific OspA fragment was produced by PCR as described above. The fragment was sequenced directly by using a cycle sequencing kit (Life Technologies GIBCO-BRL, Gaithersburg, MD). Sequence construction was facilitated by using the SEQED program (University of Wisconsin Genetics Computer Group), and sequence alignments were done by using the GAP sequence alignment program (University of Wisconsin Genetics Computer Group) in the Mayo Foundation Research Computing Facility. GAP and length weights were assigned at 5.00 and 0.3, respectively. The FASTA algorithm was used to identify similar sequences in the GenBank data base.

Results

Geographic distribution of archived specimens. The 280 ear skin punch biopsy samples were collected from *Peromyscus* species and red-backed vole (*Clethrionomys gapperi*) study skins, as well as a few from chipmunks (*Tamias stria-*

Downloaded from http://jid.oxfordjournals.org/ at Penn State University (Paterno Lib) on September 15, 2016

tus) and meadow voles (*Microtus pennsylvanicus*) that were found in the collections at MCZ, SI, and AMNH (table 1). Many of the specimens were collected from areas known to be highly endemic today: Cape Cod, Massachusetts, and Montauk, Shelter Island, and Westchester County, New York. The MCZ collection contained specimens representing many parts of the northeastern United States, whereas specimens from other areas of the country were identified in the SI and AMNH collections. Several sets of specimens were intended as negative controls: 15 samples from Emigrant Gulch, Montana, and 5 samples from Stevensville, Montana, were collected from a state with no reported cases of locally acquired Lyme disease [5]. Other large groups of specimens were from currently nonendemic areas in states with reported cases of Lyme disease; an example of the latter is the collection from Fort Snelling, Minnesota (table 1).

Detection of B. burgdorferi DNA in archived tissues. We have used a PCR-based system to detect *B. burgdorferi* in inbred mice in ear skin samples obtained after 1 year of infection [6]. In the latter experiments, detection of the gene encoding OspA demonstrated the greatest sensitivity. However, this PCR-based detection system had never been challenged with dried, preserved ear skin specimens from animals processed by taxidermy techniques. Accordingly, we tested dried, borax-treated specimens from known infected and uninfected animals. The sensitivity of detection in treated specimens was somewhat lower than expected; only 2 of 5 infected animals were PCR-positive (figure 1A). All 5 negative controls were negative. These data suggested that the sensitivity of PCR for detection of OspA in treated skin specimens is lower than in untreated *P. leucopus* specimens (data not shown) or in fresh biopsy specimens from inbred mice but that it could still be useful as a survey technique if enough specimens were analyzed.

Two of the 280 archived specimens were reactive for the *B. burgdorferi* OspA target sequence (figure 1B) in three separate analyses of the original ear skin digest. One specimen was collected from West Dennis, Massachusetts, in May 1894 and the other from South Dennis, Massachusetts, in June 1894. The remaining 280 specimens, representing present-day endemic and nonendemic areas, were negative in repetitive analyses. Of 56 "no target" negative controls run in parallel, none yielded a positive result. Likewise, of the 20 samples collected from Montana that were intended as negative controls, none were positive. Two attempts to detect the 16S ribosomal DNA target [3] in the 2 OspA-positive specimens were unsuccessful, most likely because of greater sensitivity of the plasmidborne targets [7].

Sequence analysis of the amplification product. To verify the identity of the amplification product from the true-positive specimens and to determine genetic sequence variation in the *ospA* gene that might have occurred over time (nearly 100 years), we determined the nucleotide sequence of the 156-bp OspA amplification product recovered from one of the animals (West Dennis, MA, 1894). DNA cycle sequencing was done on bulk-amplified PCR product directly to minimize the effects of random Taq polymerase incorporation errors. Alignment of the region between the two PCR primers (comprising 107 bp) showed that the sequence derived from the infected *Peromyscus* tissue collected from West Dennis in 1896 was identical to that of type strain B31 and is thus representative of most North American isolates of *B. burgdorferi* examined to date (data not shown).

Discussion

The absence of North American reports of erythema migrans before 1970, well described at the turn of the century in Europe [2], appears to indicate that Lyme disease is a recently introduced zoonosis. Increases in European immigration may have introduced *B. burgdorferi* with domestic animals or with Norway rat (*Rattus norvegicus*) infestations. Alternatively, a tickborne agent could easily have been introduced to the United States by the active trade in game ani-

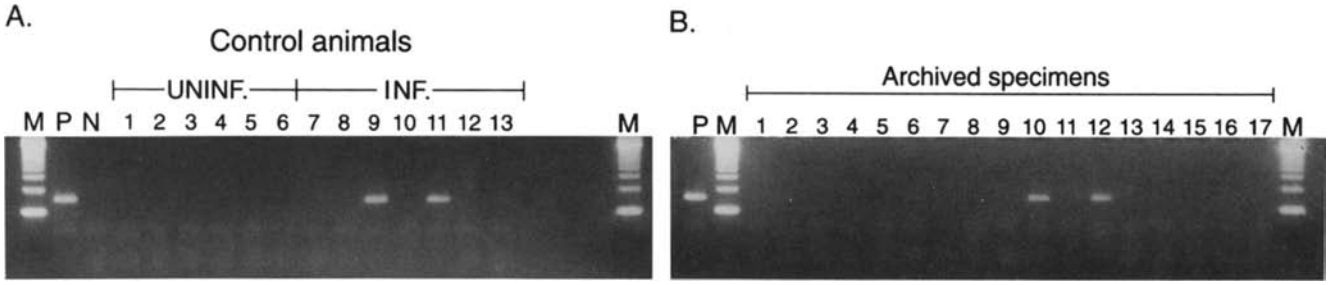

Figure 1. **A**, Detection of *B. burgdorferi* DNA in modern-day skin specimens treated with borax, alum, and arsenic. Five infected *P. leucopus* specimens trapped in 1990 on Nantucket Island (xenodiagnosis confirmed) and 5 uninfected colony-reared mice were tested. Shown are duplicate analyses of 2 infected mice (lanes 7 and 8, lanes 12 and 13) and 1 uninfected mouse (lanes 5 and 6). P, positive control (strain N40); N, negative control; M, 123-bp DNA ladder. **B**, Analysis of 17 archived specimens from Harvard Museum of Comparative Zoology obtained in Massachusetts: lane 1, Hudson, February 1870; lanes 2 and 3, Hudson, May 1870; lanes 4–6, Cambridge, November 1892; lanes 7 and 8, Muskeget, June 1893; lane 9, West Tisbury, June 1893; lane 10, West Dennis, June 1894; lanes 11–14, South Dennis, May 1894; lanes 15–17, negative controls. P and M same as in **A**.

Downloaded from http://jid.oxfordjournals.org/ at Penn State University (Paterno Lib) on September 15, 2016

mals. Hundreds of European hares (*Lepus europaeus*) from Hungary, for example, were deliberately introduced in Dutchess County, New York, over a 15-year period starting in the late 1890s [8]. Our own studies of skin biopsies from Mayo Clinic patients with a diagnosis of acrodermatitis chronica atrophicans suggest that human Lyme disease existed in the United States as early as 1912, but all of the patients with this disorder were European immigrants [9].

Cases of *I. dammini*–associated erythema migrans or human babesiosis were simultaneously recorded from the upper Midwest [10] and coastal New England [11] in 1969, and Lyme disease was described in a California patient soon thereafter [12]. Although multiple introductions cannot be excluded for the origin of the North American Lyme disease epidemic, such events taking place in these three geographically diverse areas, with Lyme disease emerging as an epidemic zoonosis within the span of a decade, appears improbable.

An alternative hypothesis is that the agent of Lyme disease has been cryptically enzootic in much of the United States even before old-world colonization but has recently emerged as an intensely zoonotic agent because of regionwide changes in the landscape, such as reforestation [13]. The description of cryptic transmission cycles within narrow host-specific *Ixodes*–small mammal associations [14, 15] provides evidence for the existence of enzootic transmission of *B. burgdorferi* in the absence of human infection. Under this hypothesis, the proliferation of aggressive human host–seeking vectors such as *I. dammini*, the widespread recreational use of wilderness sites, and the preference of many residents of endemic areas for wooded homesites have all converged to increase human exposure.

We have tested this hypothesis by analyzing museum specimens of vector and reservoir hosts for evidence of spirochetal DNA. In a previous study, evidence of infection was detected by PCR in alcohol-preserved ticks collected during the 1940s, thereby demonstrating the presence of the agent nearly 30 years before the first reports leading to the formal recognition of the disease. Ticks collected earlier than this period, however, were not found during a search of major museum collections. Accordingly, we turned to collections of the main reservoir in the northeastern United States, the white-footed mouse, in an attempt to extend our observations. By concentrating our efforts on *P. leucopus* and on other select rodents known to be competent reservoirs of the agent of Lyme disease, taken from sites that were recognized as intensely zoonotic in the early years of the emerging epidemic (coastal New England and northwest Wisconsin), we attempted to maximize our chances of detecting evidence of infection. Such sites may represent ancestral foci where ecologic conditions have allowed for the long-term perpetuation of *B. burgdorferi* [16]. States that now rarely report bona fide human cases served as our controls, with the reasoning that such sites were less likely to have had intense enzootic transmission before the 1960s. We were gratified that the 2 positive mouse specimens were collected within a radius of a few miles, in an area where Lyme disease has most likely been zoonotic for decades.

We are acutely aware of the potential for false-positive results in PCR assays. We addressed the issue of contamination with stringent amplicon contamination–control procedures and inclusion of multiple negative reagent controls [4]. Instruments used for removing skin samples from museum specimens were autoclaved before use and flamed before sampling each specimen. Microcentrifuge tubes used to transport skin samples were from unopened bags of new tubes. We believe that the geographic and temporal clustering of the 2 positive mouse samples argues against a contamination artifact. The two Dennis, Massachusetts, study skins that contained spirochetal DNA were maintained in a single museum cabinet drawer with other Cape Cod specimens collected during the 1890s (some of which were analyzed and found to be negative) and to our knowledge have never been associated with contemporary specimens that may have been a source of contamination. The absence of evidence of infection in specimens from West Virginia, Montana, and North Carolina, in particular, argues against the inadvertent transfer of nucleic acids from some Massachusetts and New York specimens stored within the same museum drawers.

The absence of detectable infection in the remainder of samples from rodents collected in other probable sites of longstanding transmission is disappointing but not surprising. Our preliminary observations on ear tissue taken from contemporary mice, which were treated with borax and dried to simulate the preparation of study skins, suggested that similar procedures used by past collectors may have had an inhibitory effect on DNA amplification. Experiments on some specimens that were not washed before digestion showed substantial inhibitory activity. Other sources of inhibition may lie in the various compounds used in annual fumigation to prevent insect damage to valuable specimens. In addition, low copy numbers of borrelial DNA may be expected within such a small sample of skin. Indeed, BSK culture of ear punch biopsies from experimentally infected C3H/HeJ mice appears to be as or more sensitive than PCR analysis of matched samples [6] because a single organism may multiply over time within the medium, but the probability of amplifying DNA from a small number of organisms within a biopsy may depend on repeated attempts to analyze the total extract. Extracting larger pieces of skin might improve the sensitivity of similar analyses by PCR in the future; however, such destructive analysis of museum specimens should be done sparingly because of the heavy demands now being placed on museums since the advent of PCR.

Our study demonstrates the presence of the agent of Lyme disease in the main northeastern United States enzootic reservoir, *P. leucopus*, before the last turn of the century. The existence of *B. burgdorferi* in North America contempor-

Downloaded from http://jid.oxfordjournals.org/ at Penn State University (Paterno Lib) on September 15, 2016

aneous with the earliest European clinical reports of various Lyme disease sequelae definitively argues against an introduction event during this century. Biogeographic considerations also suggest an ancient origin for the agent of Lyme disease [16], but such a hypothesis may remain largely untestable.

Acknowledgments

We acknowledge the invaluable assistance of Maria Rutzmoser (Harvard MCZ); Rob Voss, Gary Musser, and Wolfgang Fuchs (AMNH); and Al Gardner, Bob Fisher, and Dick Thorington (SI).

References

1. Afzelius A. Verhandlungen der Dermatologischen Gesellschaft zu Stockholm. Arch Dermatol Syphilol **1910**;101:404.
2. Steere AC, Malawista SE, Snydman DR, et al. Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three Connecticut communities. Arthritis Rheum **1977**;20:7–17.
3. Persing DH, Telford SR, Rys PN, et al. Detection of *Borrelia burgdorferi* DNA in museum specimens of *Ixodes dammini* ticks. Science **1990**;249:1420–3.
4. Rys PN, Persing DH. Preventing false positives: quantitative evaluation of three protocols for inactivation of polymerase chain reaction amplification products. J Clin Microbiol **1993**;31:2356–60.
5. Centers for Disease Control. Lyme disease—United States 1987 and 1988. Morb Mortal Wkly Rep **1989**;38:668–76.
6. Fikrig E, Barthold SW, Persing DH, Sen X, Kantor FS, Flavell RA. *Borrelia burgdorferi* strain 25015: characterization of OspA and vaccination against infection. J Immunol **1992**;148:2256–60.
7. Persing DH, Rutledge BJ, Rys PN, et al. Target imbalance: disparity of *Borrelia burgdorferi* genetic material in synovial fluid from Lyme arthritis patients. J Infect Dis **1994**;169:664–8.
8. Godin AJ. Wild mammals of New England. Baltimore: Johns Hopkins University Press, **1977**.
9. DiCaudo DJ, Su WPD, Marshall WF, Malawista SE, Barthold SW, Persing DH. Acrodermatitis chronica atrophicans in the United States: clinical and histopathologic features of six cases. Cutis **1994** (in press).
10. Scrimenti RJ. Erythema chronicum migrans. Arch Dermatol **1970**;102:104.
11. Western KA, Benson GD, Gleason NN, Healy GR, Schultz MG. Babesiosis in a Massachusetts resident. N Engl J Med **1970**;283:854–6.
12. Campagna J, Lavoie PE, Birnbaum NS, Furman DP. Lyme disease in northern California. West J Med **1983**;139:319–23.
13. Spielman A, Wilson ML, Levine J, Piesman J. Ecology of *Ixodes dammini*–borne human babesiosis and Lyme disease. Annu Rev Entomol **1985**;4:408–14.
14. Telford SR III, Spielman A. Enzootic transmission of the agent of Lyme disease in rabbits. Am J Trop Med Hyg **1989**;41:482–90.
15. Brown RN, Lane RS. Lyme disease in California: a novel enzootic transmission cycle of *Borrelia burgdorferi*. Science **1992**;256:1439–42.
16. Telford SR III, Gorenflot A, Brasseur P, Spielman A. Babesial infections in humans and wildlife. In: Kreier JP, ed. Parasitic protozoa. 2nd ed. Vol 5. New York: Academic Press. **1993**:1–47.

Downloaded from http://jid.oxfordjournals.org/ at Penn State University (Paterno Lib) on September 15, 2016