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SPIROCHETES IN *IXODES DAMMINI* AND MAMMALS FROM CONNECTICUT

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Abstract. Spirochetes were observed in the midguts of 35% of 147 motile *Ixodes dammini* from three locations in Lyme and East Haddam, Connecticut. Positive ticks were removed from eastern chipmunks (*Tamias striatus*), raccoons (*Procyon lotor*), white-footed mice (*Peromyscus leucopus*), and a red squirrel (*Tamiasciurus hudsonicus*). Spirochetes were isolated in fortified Kelly's medium from nine questing or partially engorged *I. dammini* adults and nymphs and from the bloods of a raccoon and a white-footed mouse. Connecticut isolates from ticks and mammals were serologically indistinguishable from the original Shelter Island, New York strain when cross-tested by immunofluorescence against their mouse antisera. Sera from eight patients diagnosed as having Lyme disease contained antibodies to spirochetes isolated from ticks and mammals. Our finding of serologically and morphologically indistinguishable spirochetes in a raccoon, white-footed mouse and ticks suggests that closely related serotypes are present in wild mammals commonly parasitized by *I. dammini,* and further supports the claim that a spirochete is involved in the etiology of Lyme disease.

Lyme disease is a systemic human illness that is usually characterized by erythema chronicum migrans (ECM) and in many patients by cardiac, neurologic and arthritic sequelae.[1–3] Originally described from patients in Connecticut, cases have more recently been reported from widely scattered areas of the United States.[4] The disease is thought to be caused by an infectious agent, possibly a spirochete, that may be transmitted by ixodid ticks such as *Ixodes dammini* and *Ixodes pacificus*.[5] Nearly ⅔ of the 126 *I. dammini* adults collected on Shelter Island, New York in October, 1981 were confirmed to harbor spirochetes by microscopic examination. These bacteria reacted in fluorescent antibody tests against convalescent sera from persons who had Lyme disease. We report here the prevalence of juvenile and adult *I. dammini* infected with spirochetes in Lyme and East Haddam, Connecticut, the isolation of these organisms from midguts of *I. dammini* and from the blood of a raccoon, *Procyon lotor,* and a white-footed mouse, *Peromyscus leucopus,* and serologic reactivity of spirochetes in indirect fluorescent antibody (IFA) tests.

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MATERIALS AND METHODS

Tick collections

Ticks were collected from April through July 1982, as they crawled on persons or dogs or after they had attached to a variety of small- and medium-sized wild mammals. Procedures for the capture and immobilization of wild animals have been described.[6]

Three sites were chosen for sampling in eastern Connecticut. Questing ticks were obtained from Nehantic State Forest in Lyme, a 776 ha tract of uninhabited, largely deciduous forested land dominated by oaks (*Quercus*). A private residence on a 3.7 ha pasture bordered by woodlands is located 5.3 km north of Nehantic State Forest and is hereafter designated as East Haddam Site No. 1. The mother at this residence had contracted Rocky Mountain spotted fever in 1981, and one of her two children was diagnosed as having Lyme disease in July 1982. East Haddam Site No. 2 is a 105 ha tract of forests and hay fields situated 8.8 km northwest of East Haddam Site No. 1. The mother and one of her two children at this site contracted Lyme disease in 1978 and 1979, respectively. Mammals at the East Haddam sites were captured along streams, hedgerows, stone

walls, in and around swamps, and in upper drier woodlands. After ticks were removed and blood samples were taken, individuals were "ear-tagged" and released into the field.

Midgut dissections and examinations for spirochetes

Live ticks were immersed in fortified Kelly's medium,[5,7,8] and the extreme caudal part of the tick's abdomen was cut off. After removal of the dorsum, the esophagus, hindgut and attached tracheae were severed, and the midgut was extracted and placed in a small drop of medium. The preparation was either overlaid with a cover slip and examined directly for spirochetes by dark-field or phase-contrast microscopy at 640 or 1000×, or indirectly by triturating the midgut with forceps, allowing it to dry for 24–48 hours, and then examining whether the preparation reacted with high titered (≥1:2,048) mouse or human antisera in indirect immunofluorescence tests.

Isolation of spirochetes

Attempts were made to isolate spirochetes from midgut tissues of ticks that had been surfaced-sterilized with hydrogen peroxide for 70 sec, rinsed in 70% ethanol, stored in sterile moist gauze pads and dissected in a laminar-flow hood. About one-third to one-half of each midgut was examined in fortified Kelly's medium by dark-field or phase-contrast microscopy to determine if the tick was infected. The remaining midgut tissues were placed into a culture tube containing 8 ml of fortified Kelly's medium and maintained at 33°C ± 2°. Samples of inoculated medium were microscopically examined for spirochetes at 1- to 2-week intervals over a 6-week period.

Sterile whole or heparinized blood was drawn from the hearts of immobilized host mammals, and within 20 minutes, 1–5 drops of blood (1 drop = 0.01 ml) were added to separate culture tubes which were held and examined for spirochetes as described above.

Serotyping spirochete isolates

Immunofluorescence procedures were essentially those reported for spotted fever-group rickettsiae.[9] Test antigens consisted of spirochetes grown in fortified Kelly's medium mixed 1:1 with 5% yolk sac diluted in phosphate buffered saline (PBS) (0.15 M Na_2HPO_4, 0.15 M KH_2PO_4, 0.15 M NaCl, pH 7.2) and containing 0.01% sodium azide. Antigens were stored at 4°C for no more than 4 weeks before use. Antiserum of each isolate was prepared in six 21- to 28-day-old Swiss mice by inoculating 0.3–0.5 ml of Kelly's medium ($n \geqq 16{,}600$ spirochetes/ml) into their tail veins on days 0 and 7. Mice were exsanguinated on days 14–18, and sera from each respective group were pooled. Antigens of seven isolates, including the original Shelter Island, New York strain,[5] were tested against homologous and heterologous antisera in reciprocal cross tests.

Antigens were applied as a thin film with the aid of a pasteur pipet to premarked microscope slides, and preparations were allowed to dry for 24–48 hours at room temperature before fixation in acetone for 10 min. Utilizing 5% yolk sac as a diluent, serial twofold dilutions of mouse sera were placed over a series of antigen preparations. Following incubation at 37°C for 30 min, slides were washed in PBS and allowed to dry. Commercially prepared fluorescein isothiocyanate-labeled rabbit anti-mouse globulin (ICN Pharmaceuticals, Inc., Cleveland, OH) diluted to 1:40 in PBS was placed over each antigen set. Slides were again incubated at 37°C for 30 min and rinsed in PBS. After drying, slides were mounted with buffered glycerol and examined with a Zeiss fluorescence microscope. End-points were identified as the highest serum dilutions for which there was distinct spirochetal fluorescence. Tests were performed with known conjugate and positive and negative serum controls.

Clinical records and serologic analyses of human sera

Clinical information and serum samples of persons diagnosed as having Lyme disease were provided by the Virology Laboratories of the Connecticut Department of Health. Commercially prepared fluorescein isothiocyanate-labeled goat antihuman Ig (Grand Island Biological Company, Grand Island, NY) and IgM (Cappel Laboratories, West Chester, PA) conjugates diluted to 1:100 in PBS were used to detect antibodies. Suspected cases were considered laboratory confirmed if serum titers ≧1:64 were recorded. Sera from persons living in or having visited southeastern Connecticut and not known to have had Lyme disease were included as controls.

TABLE 1

Prevalence of Ixodes dammini *infected with spirochetes in Lyme and East Haddam, Connecticut*

Collection site	Stages of development	Host mammal or questing	Fresh preparations		IFA preparations	
			No. exam.	No. (%) pos.	No. exam.	No. (%) pos.
Lyme (Nehantic State Forest)	Female	Questing	22	10 (45)		
	Male	Questing	15	4 (27)		
	Nymph	Questing	1	0		
East Haddam Site No. 1	Male	Questing	9	5 (56)		
	Female	Questing	13	7 (54)		
	Nymph	Eastern chipmunk	2	2 (100)		
	Nymph	Raccoon	5	2 (40)	7	0
	Nymph	White-footed mouse	0	0	1	0
	Larva	White-footed mouse	1	0	2	2 (100)
East Haddam Site No. 2	Nymph	Virginia opossum	5	0	1	0
	Larva	Virginia opossum	1	0		
	Nymph	Raccoon	10	0	10	1 (10)
	Nymph	White-footed mouse	13	4 (31)	4	1 (25)
	Larva	White-footed mouse	5	1 (20)	11	4 (36)
	Nymph	Red squirrel	1	1 (100)		
	Male	Questing	1	1 (100)		
	Female	Questing	1	1 (100)		
	Nymph	Eastern chipmunk	4	4 (100)	2	1 (50)

RESULTS

Larval, nymphal and adult *I. dammini,* obtained from Lyme and East Haddam contained spirochetes. Prevalence of infection for juvenile and adult *I. dammini* collected in the Nehantic State Forest and at East Haddam sites No. 1 and No. 2 were 37, 45, and 27%, respectively (Table 1). Positive ticks were removed from eastern chipmunks (*Tamias striatus*), raccoons, white-footed mice, and a red squirrel (*Tamiasciurus hudsonicus*). An additional 12 nymphal or adult *I. dammini* collected in Voluntown, Westbrook, Chester, East Lyme, Old Lyme and two additional locations in East Haddam, Connecticut were negative for spirochetes. *Dermacentor variabilis* (n = 41), *Ixodes cookei* (n = 3) and *Ixodes texanus* (n = 9) collected from Lyme and East Haddam were also negative.

Spirochetes were observed in freshly prepared wet preparations of midguts and in similar tissue smears stained by immunofluorescence (Table 1). From the two East Haddam sites where ticks were examined by both techniques, nine (23.7%) of the 38 *I. dammini* examined by immunofluorescence were positive while 28 (39.4%) of the 71 ticks examined in wet preparations contained spirochetes.

Infected *I. dammini* tended to be clustered on host animals. For example, seven of eight nymphs removed from three chipmunks were infected, but no spirochetes were found in seven ticks parasitizing two Virginia opossums (*Didelphis virginiana*) (Table 2). Only two of 15 raccoons were bearing positive ticks, but two of the three ticks harboring spirochetes were feeding on the same animal along with three negative ticks. The other positive tick from a raccoon was the only specimen examined from that particular host. Six white-footed mice were carrying 12 infected larval and/or nymphal *I. dammini*. The remaining 25 *I. dammini* engorging on 16 other white-footed mice were negative.

Spirochetes were isolated from the midguts of

TABLE 2

Number of mammals captured at East Haddam sites No. 1 and No. 2 which were parasitized by spirochete-infected Ixodes dammini

Host animal	No. of hosts with ticks	No. of hosts with infected ticks	No. positive ticks/total ticks examined
Eastern chipmunk	3	3	7/8
Virginia opossum	2	0	0/7
Raccoon	15	2	3/35
White-footed mouse	22	6	12/37
Red squirrel	1	1	1/1

TABLE 3
Spirochete isolations from Connecticut ticks and mammals

CT number	Isolation source	Collection	
		Date	Site
2349	*I. dammini* questing female	2 May	Lyme
2356	*I. dammini* nymph feeding on white-footed mouse	18 May	East Haddam Site No. 2
2371	*I. dammini* questing male	18 May	East Haddam Site No. 1
2372	*I. dammini* questing female	18 May	East Haddam Site No. 1
2384	*I. dammini* questing female	24 April	Lyme
2397	*I. dammini* questing female	10 June	Lyme
2404	*I. dammini* questing female	15 June	East Haddam Site No. 1
2475	*I. dammini* nymph feeding on eastern chipmunk	15 June	East Haddam Site No. 1
2497	*I. dammini* nymph feeding on eastern chipmunk	15 June	East Haddam Site No. 2
2535	Heparinized blood of raccoon	30 June	East Haddam Site No. 2
2591	Whole blood of white-footed mouse	15 July	East Haddam Site No. 2

questing and partially engorged *I. dammini* (Table 3). Portions of midguts from 16 questing females, eight questing males, and 13 engorging nymphs, known to be infected with spirochetes by examination of wet preparations, were inoculated into fortified Kelly's medium. Four isolations were made from individual adult ticks (3 female and 1 male). Cultures were also established from three partially engorged *I. dammini* nymphs. Midguts of an additional 13 females, four males, 24 nymphs and seven larvae initially considered negative for spirochetes were also placed separately in the culture medium and two isolations were made from questing females.

Bloods from 79 white-footed mice, one Norway rat (*Rattus norvegicus*), 21 raccoons, one meadow vole (*Microtus pennsylvanicus*), one Virginia opossum, one woodland jumping mouse (*Napaeozapus insignis*), five gray squirrels (*Sciurus carolinensis*), and five eastern chipmunks, were inoculated into fortified Kelly's medium. Spirochetes were isolated from one raccoon and one white-footed mouse (Table 3), and were of similar size and appearance to those recovered from *I. dammini*. Connecticut spirochetes varied in length from 13.9 to 36.3 μm. Mean lengths ($n = 10$) of our fresh isolates ranged from 19.9 ± 3.8 μm for CT 2372 isolate to 25.4 ± 6.2 μm for CT 2472, and were greater than measurements of the Shelter Island isolate ($\bar{x} = 15.6 \pm 2.9$ μm) which had been in culture for 10 months.

Isolates were serologically indistinguishable. For example, in reciprocal cross tests using Swiss mouse antisera, the Shelter Island, New York spirochetes and six Connecticut isolates (4 from ticks and 2 from mammalian blood) had similar homologous and heterologous titration end-points (Table 4). The relatively low-titered reaction of the Shelter Island antigen with CT 2384 antisera is attributed to variation in the test.

Seven Connecticut residents and one visitor from California, all with a history of diagnosed Lyme

TABLE 4
Serotypic results of Connecticut spirochete isolates by indirect fluorescent antibody tests

Mouse antisera to isolate	Antigens						
	Shelter Island, N.Y.	CT 2349	CT 2356	CT 2384	CT 2475	CT 2535	CT 2591
Shelter Island, N.Y.	32,768*	32,768	16,384	32,768	32,768	32,768	32,768
CT 2349	16,384	32,768	16,384	32,768	16,384	32,768	32,768
CT 2356	16,384	32,768	32,768	32,768	16,384	32,768	32,768
CT 2384	8,192	32,768	32,768	32,768	16,384	32,768	32,768
CT 2475	32,768	32,768	32,768	32,768	32,768	32,768	32,768
CT 2535	32,768	32,768	16,384	32,768	32,768	32,768	32,768
CT 2591	32,768	32,768	32,768	32,768	32,768	32,768	32,768

* Titers are expressed as the reciprocal of end-point dilutions.

TABLE 5

Indirect immunofluorescence antibody titers of sera from eight persons exhibiting symptoms of Lyme disease against seven spirochete antigens isolated from Ixodes dammini, *a raccoon and a white-footed mouse*

				IFA titers* to spirochete isolates							
				Ig conjugate							IgM conjugate
Patient	Disease onset	Illness day	Day antibiotics first administered†	Shelter Island	CT 2349	CT 2356	CT 2384	CT 2475	CT 2535	CT 2591	CT 2356
A.S., Age 49, ♂	6- 9-80	17	3	128	128	256	1,024	2,048	1,024	1,024	512
K.F., Age 15, ♀	8-20-80	‡	None	32,768	16,384	32,768	32,768	32,768	32,768	ND‖	ND
R.S., Age 55, ♂	6-10-81	6	9	64	64	64	256	128	128	256	128
		43		64	256	64	256	128	256	256	128
B.P., Age 39, ♀	7-15-81	8	12	64	64	64	256	128	128	128	128
		19		128	256	256	512	512	256	256	128
M.P., Age 31, ♀	8-29-81	6	§	2,048	4,096	2,048	16,384	8,192	16,384	2,048	2,048
R.M., Age 43, ♂	7-11-82	25	19	1,024	1,024	1,024	2,048	512	512	2,048	128
S.M., Age 20, ♂	7- 9-82	8	8	128	128	128	128	256	128	512	128
D.B., Age 11, ♂	7-23-82	9	21	2,048	512	512	512	512	512	1,024	0
		21		1,024	ND	512	1,024	1,024	1,024	2,048	0

* Titers are expressed as the reciprocal of end-point dilutions.
† Penicillin or tetracycline.
‡ ECM was not observed in this patient. She was admitted to the hospital with swollen knee joints on 20 August 1980.
§ Penicillin was administered after the serum sample was drawn, but the specific date is unknown.
‖ Not done.

disease, contained antibodies against spirochetes (Table 5). The eight individuals lived in or had visited areas inhabited by *I. dammini*; two remembered being bitten by a tick. Erythema chronicum migrans appeared in six persons (B.P., A.S., M.P., R.M., D.B., S.M.), and swelling of knee joints occurred in K.F. Myalgia, malaise, fatigue, fever and headache developed in all individuals except K.F. Four persons exhibited arthralgia (D.B., R.M., R.S., M.P.) and backache (D.B., B.P., S.M., R.S.), and five presented with stiff neck (D.B., A.S., B.P., R.S., S.M.). Ig serum titers ranged from 1:64 to 1:32,768 (Table 5), but differences in end-points did not exceed 8-fold (except in 1 sample, A.S.) for any given serum sample when tested against seven different isolates. The highest titer (1:32,768) was obtained in a person (K.F.) who exhibited acute arthritis and who had not received antibiotics at the time the serum was drawn. More modest titers were recorded for the other sera. In four instances (A.S., R.S., B.P., R.M.), tetracyclines or penicillin had been administered prior to sampling, and antibody titers were lower. Significant IgM antibody was detected in six of seven patients against the CT 2356 isolate (Table 5). Sera from 16 patients without a history of ECM or arthritic disorders were nonreactive with spirochete antigens.

DISCUSSION

Sampling sites were selected in Lyme and East Haddam because of the high prevalence of Lyme disease among humans.[2,3] Although infection rates for *I. dammini* were lower than those reported for Shelter Island, New York ticks, our data support the hypothesis that a spirochete is involved in the etiology of Lyme disease.[5]

Spirochetes may be detected in ticks by examining fresh smears of midgut contents, by staining procedures, and by culturing spirochetes in artificial medium. Since two isolations of spirochetes were made from ticks initially considered negative (by examination of freshly prepared midgut tissues), we believe that our rates of infection are conservative. Although spirochetes grow in fortified Kelly's medium, isolations from known infected midguts (by microscopic examination) were successfully cultured only 20% of the time, indicating that even a greater percentage of ticks identified as negative may have been infected when evaluated by the latter method.

Our finding of serologically and morphologically indistinguishable spirochetes in a raccoon, white-footed mouse, and in ticks suggests that closely related serotypes are present in wild mammals commonly parasitized by *I. dammini*. The clus-

tering of infected ticks on certain mammals, the presence of infected engorged or partially engorged larvae, and isolations of spirochetes from the blood of wild mammals also suggest that *I. dammini* may acquire these bacteria by feeding on vertebrate hosts.

Spirochetes have not yet been isolated from humans afflicted with Lyme disease. Antigens from our six Connecticut and the Shelter Island isolates reacted with acute and convalescent sera from persons diagnosed as having Lyme disease but were non-reactive with sera from humans with no known history of this illness. The presence of significant, detectable quantities of IgM antibody in six of seven persons showing symptoms of Lyme disease suggests recent patient exposure to these spirochetes, and further corroborates the hypothesis that these organisms may be the etiologic agent of the disease.[5] We are unable to explain the absence of IgM antibody in one person. Possibly, this individual previously had been sensitized to a similar antigen without reappearance of IgM globulin. Our inability to demonstrate a rise in titer in paired sera may be attributed in part to the presence of antibody at the time of onset of illness, which may occur 3–30 days after tick bite[4] or exposure to spirochetes. High titers ($\geqq 2{,}048$) may occur in patients not treated with antibiotics.

Standardized procedures for isolating ixodid-tick associated spirochetes from mammals have not yet been developed, but our two isolations from heparinized and whole blood suggest that the placement of small quantities of infected blood into culture tubes will yield isolations. Since we do not know how many of the blood samples were actually infected, we are unable to determine prevalence of infection in wild hosts or to estimate the success of this procedure. Nonetheless, these methods of isolating spirochetes from wild mammals may also prove useful in their recovery from man.

Ixodes dammini is a three-host tick that parasitizes birds and small-, medium- and large-sized mammals.[6, 10–14] It may take 2 years to complete its life cycle, and questing adults are typically most abundant in late fall and spring. Nymphs are also active in spring, whereas larvae reach their peak in late summer and early fall. Although spirochetes are maintained trans-stadially in *I. dammini,* we do not know if they are passed transovarially. However, the ticks *I. pacificus,* the incriminated vector of Lyme disease in the western United States, and *Ixodes ricinus,* the vector of erythema chronicum migrans in Europe (Burgdorfer, unpublished data) have been shown to transovarially transmit spirochetes indistinguishable from those associated with *I. dammini.* Thus, it appears that unfed *I. dammini* larvae, in addition to possible transovarial passage, may acquire the spirochete when they feed on infected hosts such as white-footed mice, and after molting, nymphs may then vector the bacteria to other maintenance or incidental hosts which may include man.

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