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Diversity of European Lyme Disease Spirochetes at the Southern Margin of Their Range

FRANZ-RAINER MATUSCHKA,[1,2] BIRTE KLUG,[1] THOMAS W. SCHINKEL,[1] ANDREW SPIELMAN,[2] AND DANIA RICHTER[1,2]*

Institut für Pathologie, Virchow-Klinikum, Medizinische Fakultät der Humboldt-Universität zu Berlin, 12249 Berlin, Germany,[1] and Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts 02115[2]

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We determined whether the genospecies diversity of Lyme disease spirochetes in vector ticks questing on a subtropical island is as broad as that in Central Europe. Although spirochetes infected <1% of the ticks sampled on Madeira Island, these infections included all three genospecies implicated in human disease. Therefore, spirochetal diversity is as great at the southern margin as it is in the center of this pathogen's range.

The agent of Lyme disease, *Borrelia burgdorferi* sensu lato, is enzootic on the subtropical island of Madeira (8). No terrestrial mammals were present until they were introduced some 600 years ago. The resulting rodent fauna now includes only house mice, Norway rats, and black rats. Free-ranging domestic or feral cats, dogs, rabbits, pigs, cattle, sheep, horses, and goats may be abundant locally. Subadult ticks frequently parasitize both kinds of rats, which appear to be the main reservoir hosts of these spirochetes on the island (8). Cattle, sheep, and dogs serve as definitive hosts for the vector tick *Ixodes ricinus*. The agent of human Lyme disease is particularly diverse on the European continent, including at least three genetically distinguishable genospecies (1, 3, 11) as well as intragenospecific serotypes (13). Fewer such human pathogens appear to be transmitted elsewhere. To the east, in Asia, *B. burgdorferi* sensu stricto appears to be absent (6, 12). On the North American continent, this flora appears to be limited to *B. burgdorferi* sensu stricto (1), and subarctic and subantarctic regions seem to support only *Borrelia garinii* (10). The diversity of spirochetes transmitted immediately south of the European center of intense transmission has not yet been characterized. If genospecies diversity varies with proximity to the European center of transmission and with the intensity of transmission, diversity would be more limited in sites that are remote and small and where infection is infrequent. Accordingly, we described the diversity of spirochete genospecies on Madeira Island, which is located at the southern margin of the known European distribution of these pathogens.

Host-seeking (questing) ticks on Madeira Island were sampled (8) from the margins of meadows in which domestic animals had pastured. We selected about a dozen such sites at altitudes varying from 500 to 1,500 m. Ferns predominate there. *I. ricinus* ticks were sampled individually by aspirating them directly from vegetation into screened vials. As many as 15 nymphal ticks generally were found in contact with each other within a few centimeters above the ground, and clusters of 3 or 4 adult ticks were found at levels up to some 50 cm higher. The fragile nature of these ferns intermixed with thorny brush rendered more conventional sampling methods impractical. Ticks were held in a water-saturated atmosphere at ambient temperature.

In our general survey of the frequency of spirochetal infection in questing ticks, each adult tick was analyzed individually and nymphs were analyzed either individually or in pools. Alternatively, pooled samples of field-derived questing nymphs were permitted to feed on individual Mongolian jirds (*Meriones unguiculatus*). To characterize the spirochetes infecting a jird, noninfected, laboratory-reared, larval *I. ricinus* ticks were permitted to engorge on each jird as early as 2 weeks after challenge. The body contents of the resulting nymphs were screened by means of dark-field microscopy to detect spirochetal infection, and a sample of ticks from all infected cohorts was analyzed by PCR. In addition, tissue samples were obtained from the kidneys of three jirds 2 weeks after field-derived nymphs had engorged on them.

To characterize spirochetes infecting ticks, posterior opisthosomas were opened, the midguts were dissected out and incubated with proteinase K (Boehringer Mannheim, Mannheim, Germany), and DNA was extracted with phenol-chloroform or a QIAamp Tissue kit (QIAGEN GmbH, Hilden, Germany) (9). *Borrelia* genospecies were characterized by amplifying and sequencing a segment of the gene encoding the outer surface protein A (OspA). Nested PCR was carried out as described elsewhere (9) with the following primer sequences (5′-3′): outer primers GGTCTAATATTAGCCTTAATAGCATG and TCAGCAGCTAGAGTTCCTTCAAG and inner primers CA TGTAAGCAAAATGTTAGCAGCC and CTGTGTATTCA AGTCTGGTTCC. For comparison, each PCR amplification series included two laboratory-reared nymphs that had fed as larvae on *Borrelia afzelii*-infected jirds and two that had fed on noninfected jirds.

Each PCR amplification product that appeared as a single band in an ethidium bromide-stained agarose gel was purified with a QIAquick Purification kit (QIAGEN). Amplified DNA fragments were directly sequenced in both directions with the inner primers by the dideoxynucleotide chain-termination method on an ABI 373 DNA sequencer according to the instructions of the manufacturer (Applied Biosystems, Foster City, Calif.). This PCR technique detects two different coinfecting spirochete genospecies even when one is five times as numerous as the other (4).

We determined how frequently the various spirochete genospecies infected ticks on Madeira Island by directly amplifying a segment of the spirochetal *ospA* gene from the midgut con-

* Corresponding author. Mailing address: Department of Immunology and Infectious Diseases, Harvard School of Public Health, 665 Huntington Ave., Boston, MA 02115. Phone: (617) 432-1796. Fax: (617) 738-4914. E-mail: drichter@hsph.harvard.edu.

TABLE 1. Genospecies diversity of Lyme disease spirochetes in nymphal and adult *I. ricinus* ticks questing on vegetation on Madeira Island

Stage of ticks sampled	No. of ticks	No. of pools	% Pools with *Borrelia* sequences	No. of sequences indicating the following genospecies:			
				B. afzelii	*B. burgdorferi*	*B. garinii*[a]	Other
Nymphal	161	161	1.2	0	1	0	1
Nymphal	369	37	0.5	0	1	1	0
Adult	165	165	0.6	0	0	1	0
Total	695	363	0.7	0	2	2	1

[a] Serotype 5.

tents of questing nymphal and adult vector ticks. Of the ticks whose DNA was amplified individually, three contained *Borrelia* sequences, including those of *B. burgdorferi* sensu stricto and *B. garinii* (Table 1). The sequence of another sample included loci that resembled one or another of these genospecies-specific sequences without evidence of double infection. When DNA of pooled nymphal ticks was amplified, two spirochetal infections were discovered, and these included sequences corresponding to those of *B. burgdorferi* sensu stricto and *B. garinii*. Both *B. garinii* sequences matched with that designated serotype 5. Although spirochetes infect less than 1% of the questing nymphal and adult ticks sampled on Madeira Island, these infections include at least two different genospecies.

To improve sensitivity, pools of approximately 50 questing nymphs were permitted to feed on jirds. Spirochetes were later harvested from the guts of nymphal ticks that had engorged as laboratory-reared xenodiagnostic larvae on these animals. *Borrelia* DNA, including sequences that corresponded to those of *B. afzelii*, *B. burgdorferi* sensu stricto, and *B. garinii*, could be amplified from about half of these pools (Table 2). The *B. burgdorferi* sensu stricto sequence was obtained from kidney tissue of one of the hosts. The *B. garinii* sequences corresponded to that designated serotype 6. Taken together, our observations demonstrate the presence of at least three *Borrelia* genospecies and two intragenospecific serotypes on Madeira Island.

TABLE 2. Genospecies diversity of Lyme disease spirochetes in jirds that served as hosts to nymphal *I. ricinus* ticks sampled while questing on vegetation on Madeira Island

Sample source and jird no.	No. of questing nymphs engorged	Detection of *Borrelia* genospecies[b]	No. sequences indicating the following genospecies:			
			B. afzelii	*B. burgdorferi*	*B. garinii*[c]	Other
Xenodiagnostic tick[a]						
1	73	0				
2	80	0				
3	61	+	1	0	0	0
4	48	0				
5	62	+	0	0	1	0
6	54	0				
7	144	+	1	0	0	0
8	96	+	0	0	1	0
9	97	+	1	0	0	0
Host tissue						
10	86	+	0	1	0	0
11	86	0				
12	107	0				
Total						
12	994		3	1	2	0

[a] Nymphal ticks had engorged as xenodiagnostic larvae on jirds.
[b] PCR analysis was performed for all cohorts of xenodiagnostic ticks in which spirochetes had been detected by means of dark-field microscopy.
[c] Serotype 6.

Relatively few Madeiran ticks are infected by Lyme disease spirochetes. Where human infection burdens public health, e.g., in North America or in Central Europe, as many as a third of nymphal vector ticks may be infected (7). The scarcity of infection on Madeira Island permits analysis of pools of ticks as though they were being analyzed individually; virtually no pool would contain more than one infected tick, and no sample contained more than one kind of spirochete. The infrequent pattern of spirochetal infection in Madeiran ticks may be reflected in the fact that no human infections among Madeiran residents have yet been described.

In spite of the scarcity of infection in ticks on Madeira Island, the spirochetes there are surprisingly diverse, including *B. afzelii* and *B. burgdorferi* sensu stricto, and at least two of the five known serotypes of *B. garinii* that occur in Central Europe (4) infect these ticks. The range of variation in Madeiran spirochetes does not differ from that reported from Europe ($P = 0.46$ by the Fisher exact test). Diversity is greatest in Central Europe and is least in North America, where only the *B. burgdorferi* sensu stricto genospecies occurs (1). Similarly, *Ixodes* ticks of the subarctic regions of Europe seem to be infected solely by *B. garinii* (2). Although only two genospecies causing human Lyme disease are present in Asia (5), the *B. garinii* spirochetes that are found there are more heterogeneous than those in Europe (6). The range of spirochetal diversity in *I. ricinus* ticks at the apparent southern margin of the distribution of these vector ticks is similar to that in their European center of distribution.

Infrequent transmission would promote local extinction of spirochete populations. If such local extinctions were common, pathogen diversity would constrict. The European-like range of diversity of spirochetes on Madeira Island, however, suggests that these infestations may be sustained by numerous repeated introductions from Europe, perhaps via migrating birds or on domestic animals. Indeed, domestic animals, including pets and livestock, frequently are transported to the island from various parts of continental Europe. The foci of spirochetal transmission on Madeira Island may not be self-sustaining.

Although the far northern portion of the European distribution of the agent of Lyme disease appears to be restricted, our sample from its apparent southern margin is diverse. As much spirochetal diversity is present at the southern margin of the European distribution of the agent of Lyme disease as is present in Central Europe. This diversity may be maintained by repeated introductions.

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