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Genetic Heterogeneity of *Borrelia burgdorferi* Sensu Lato in *Ixodes ricinus* Ticks Collected in Belgium

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***Borrelia burgdorferi* sensu lato (s.l.), the etiological agent of Lyme disease, is transmitted by the bite of *Ixodes ricinus*. Four hundred eighty-nine ticks, collected in four locations of a region of southern Belgium where Lyme disease is endemic, were examined for the presence of the spirochete. In a PCR test with primers that recognize a chromosomal gene of all strains, 23% of the ticks were found to be infected. The species *B. burgdorferi* s.l. comprises at least three pathogenic genomospecies, *B. burgdorferi* sensu stricto (s.s.), *Borrelia garinii*, and *Borrelia afzelii*, which could be distinguished in PCR tests with species-specific primers that correspond to distinct plasmid sequences. *B. garinii* was most prevalent (53% of infected ticks), followed by *B. burgdorferi* s.s. (38%) and *B. afzelii* (9%). Of the infected ticks, 40% were infected with a single species, 40% were infected with two species, and 5% were infected with all three species. For 15% of the ticks, the infecting species could not be identified. No difference in rates of prevalence was observed among the four locations, which had similar ground covers, even though they belonged to distinct biogeographic regions. A greater heterogeneity of spirochetal DNA in ticks than in cultured reference DNA was suggested by a comparison of the results of PCRs with two different sets of species-specific primer sequences.**

The etiological agent of Lyme borreliosis, *Borrelia burgdorferi* sensu lato (s.l.), is transmitted in Europe by the bite of *Ixodes ricinus* (Acari: Ixodidae) (6). *B. burgdorferi* s.l. organisms make up a heterogeneous group of spirochetes which has been divided into at least five genomospecies, three of which are pathogenic: *B. burgdorferi* sensu stricto (s.s.), *Borrelia garinii*, and *Borrelia afzelii*. Two recently discovered genomospecies, *Borrelia japonica* and *Borrelia andersonii*, have not been isolated from Lyme disease patients (10, 16). Differences in chromosomal and plasmid sequences and studies of outer membrane proteins led to this classification (3, 5, 7, 12–15, 25, 26, 33, 35).

Lyme borreliosis exhibits a broad array of clinical manifestations: skin disorders (like erythema migrans and acrodermatitis chronica atrophicans), carditis, arthritis, and neurological symptoms. The patterns of disease in Europe and the United States appear to differ. Acrodermatitis chronica atrophicans and neuroborreliosis are more common in Europe, whereas arthritis appears to be prevalent in the United States (32). The clinical outcome seems to depend on the infecting genomospecies. Lyme arthritis has been attributed to infection by *B. burgdorferi* s.s., neuroborreliosis has been attributed to *B. garinii*, and acrodermatitis chronica atrophicans has been attributed to *B. afzelii* (1, 2, 8, 34).

Information concerning the prevalence of *Borrelia* spirochetes in tick populations is an essential part of epidemiological surveys of Lyme disease in regions of endemicity. The identification of the different genomospecies in one particular region might help to anticipate the clinical outcomes of infected persons and to evaluate the potential of a given genomospecies to infect. In screening tests for the presence of spirochetes in the arthropod vector, targets used for the amplification by PCR of *B. burgdorferi* s.l. DNA range from chromosomal loci (a surface-exposed 66-kDa protein, flagellin, and 16S rRNA) to sequences carried by plasmids (8, 15, 18, 22, 25, 27).

The aim of this study was to investigate the prevalence of each genomospecies of *B. burgdorferi* s.l. in *Ixodes ricinus* in a southern region of Belgium where Lyme disease is endemic and to evaluate the heterogeneity of strains in nature by the use of two different species-specific primer sets.

Prevalence of ticks in four geographic areas. In 1996, during the month of July (which corresponds to the period of maximum activity of the tick), 489 *I. ricinus* ticks were collected by dragging a blanket over the vegetation on the ground in Matagne-la-Petite, close to Philippeville, Namur Province, Belgium (Table 1). Two hundred fourteen square meters (corresponding to 470 blanket sweeps) was examined for the presence of *I. ricinus* in each site. The four different geographic areas yielded similar numbers of ticks, amounting on average to 0.6 tick/m^2 (no significant difference in numbers by the χ^2 test; $P > 0.3$). The four prospected sites differ geologically and thus in their forest cover. It is well known that a main limiting factor of tick survival is the hygrometry of their microhabitat (11). A high humidity can be maintained within the vegetal litter covering the soil, provided it is sufficiently thick to buffer hygrometric variations. This was the case in each of prospected sites, so that significant differences in the numbers of ticks were actually unexpected.

Infection of ticks by different *B. burgdorferi* genomospecies. Immediately after collection, the ticks were immersed in 70% ethanol and stored at 4°C until use. The ticks were dried and incubated in 100 μl of TE (10 mM Tris [pH 7.8], 1 mM EDTA) containing 200 μg of proteinase K per ml. After overnight incubation at room temperature, they were crushed with a pipette tip, boiled for 10 min, and then placed on ice for 10 min. The samples were centrifuged for 10 min at 13,000 × *g*, and the supernatants were collected and stored at −20°C. A

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TABLE 1. Descriptions of the four prospected sites

Region of Belgium	Site	Locality	UTM[a] coordinate	Dominant trees	Soil	Subsoil
Condroz	Bois de Bonne Fontaine	Vodelée	FR 235599	*Fagus sylvatica* (beech), *Carpinus betulus* (hornbeam)	Leaf litter, ivy	Sandstone
Fagne	Bois des Fagnes	Doische	FR 245585	*Quercus robur* (oak), *Carpinus betulus* (hornbeam)	Leaf litter, bramble	Schist
Calestienne	Bois de Matignolle	Treignes	FR 188513	*Fagus sylvatica* (beech)	Leaf litter	Calcareous rock
Ardenne	Chêne à l'Image	Vierves-sur-Viroin	FR 172483	*Quercus petraea* (oak), *Betula pendula* (birch)	Leaf litter, fern	Siliceous rock

[a] UTM, Universal Transverse Mercator (global coordinate system).

5-μl amount of each supernatant was added to the PCR buffer, and amplification was obtained and analyzed as described previously (18). All samples were first amplified in the presence of the c and c′ primers of Rosa et al. (30), which correspond to a highly conserved chromosomal gene supposedly present in all *B. burgdorferi* s.l. strains. Of a total of 489 ticks (444 nymphs and 45 adults) (Table 2), *B. burgdorferi* s.l. was detected in 23% of the ticks examined, and detection ranged from 20 to 26% at the various sites (no statistical difference; $P > 0.6$). At three sites, adult ticks appeared to be more infected than nymphs. Of 22 locations in the same Belgian province along the Meuse and Sambre valleys, 14 showed the presence of infected ticks (4), stressing the endemicity of Lyme disease in the southern part of Belgium.

Positive samples were next amplified in the presence of species-specific primers, designed on the basis of sequences corresponding to different plasmids belonging to the three spirochete genomospecies (18). Inhibition of the DNA polymerase in PCR-negative tick extracts was tested by adding spirochetal DNA to the reaction mixture. As shown in Table 2, *B. garinii* was most abundant (53% of infected ticks), followed by *B. burgdorferi* s.s. (38%) and *B. afzelii* (9%). These proportions correspond to results obtained in other European countries (19, 21, 34, 36). In a previous study (8), the three genomospecies of *B. burgdorferi* s.l. were shown to infect Belgian patients in a proportion corresponding to what we now find in ticks. The three genomospecies seem to be equally distributed among the four sites ($P > 0.3$). This is at variance with observations made in Valais (Switzerland) and The Netherlands, where the proportions of the three genomic groups were not the same in different areas (19, 23).

Only one species of *B. burgdorferi* s.l. was found in 40% of infected ticks, whereas two species were found in 40% and all three species were found in 5% of the ticks (with species being undetermined in 15% of the infected ticks [see below]). Infection by a single genomospecies was prevalent in one region (Ardenne), whereas infection by multiple genomospecies prevailed in another region (Calestienne) ($P < 0.01$). Mixed infections were associated mainly with *B. garinii* and *B. burgdorferi* s.s. These results for ticks were in agreement with the results of others (9, 24, 28, 29) and are also reflected in the fact that the same proportion of mixed infections occurs in patients (8).

Of the 114 ticks that scored positive with the c and c′ primers, 17 (15%) showed no amplification product with any of the three recently developed plasmid species-specific primers (18). When we amplified the same tick extracts with previously described *ospA*-derived species-specific primers (8), the number of unassigned species among infected ticks rose to 51% (result not shown). This is not due to inhibition of DNA polymerase in tick extracts, since spiked samples gave amplification products. Both primer sets were species specific, since for samples that gave an amplification signal with the *ospA* primers, the genomospecies assignment confirmed the results reported in Table 2, which were obtained with the recently described plasmid primers (18).

In all biological fluids of patients diagnosed with the c and c′ primers, the genomic group could be identified with the *ospA*-derived primers (8). With the same primers in the present study, the genomospecies could not be assigned for 51% of infected ticks, whereas with different plasmid species-specific primers, the genomospecies could not be assigned for 15% (18). This may be due to the absence of plasmids or mutations in the primer binding sequences. In any case, our results imply that the *ospA* gene might be more divergent than the three species-specific plasmid sequences and point to a wider genetic heterogeneity of *Borrelia* spp. in ticks than in humans. Similar conclusions were reached by different approaches and in several countries (17, 19, 36). Culturing of *B. burgdorferi* might also select specific genotypes among the ones able to infect ticks (20).

The amplification products obtained with the *B. garinii* primers and 12 ticks were sequenced (31) and compared with the N34 cloned plasmid sequence which had allowed the design of the species-specific primers (18). The aligned sequences over a limited region were identical to the N34 *B. garinii* sequence (not shown). This sequencing (which confirmed that the am-

TABLE 2. Detection by PCR of genomic groups of *B. burgdorferi* s.l. in *Ixodes* ticks

Area[a]	Stage[b]	Tick infection rate[c]		No. of ticks with spirochetes in genomic group[d]:		
		No. of ticks tested	No. positive (%)	Ss	Ga	Af
Co	N	97	18 (19)	14	14	0
	A	8	3 (38)	1	3	0
Subtotal		105	21 (20)	15	17	0
F	N	116	32 (28)	16	22	4
	A	12	0 (0)			
Subtotal		128	32 (25)	16	22	4
Ca	N	109	24 (22)	11	17	6
	A	23	10 (43)	9	10	2
Subtotal		132	34 (26)	20	27	8
A	N	122	25 (20)	5	13	2
	A	2	2 (100)	0	0	0
Subtotal		124	27 (22)	5	13	2
Total		489	114 (23)	56	79	14

[a] Co, Condroz; F, Fagne; Ca, Calestienne; A, Ardenne.
[b] N, nymph; A, adult.
[c] Infection rate determined by PCR with the primers of Rosa et al. (30).
[d] Genomic groups were determined by PCR with species-specific primers (18).
Ss, *B. burgdorferi* s.s.; Ga, *B. garinii*; Af, *B. afzelii*.

plified products originated from *B. burgdorferi* DNA templates) stresses the possibility that the strains in ticks which could not be identified by species-specific primers belong to genetically distinct species.

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