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Distinct Combinations of *Borrelia burgdorferi* Sensu Lato Genospecies Found in Individual Questing Ticks from Europe

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The genetic diversity of *Borrelia burgdorferi* sensu lato was assessed in individual adult *Ixodes ricinus* ticks from Europe by direct PCR amplification of spirochetal DNA followed by genospecies-specific hybridization. Analysis of mixed infections in the ticks showed that *B. garinii* and *B. valaisiana* segregate from *B. afzelii*. This and previous findings suggest that host complement interacts with spirochetes in the tick, thereby playing an important role in the ecology of Lyme borreliosis.

Borrelia burgdorferi sensu lato is a bacterial species complex comprising 10 named genospecies and several genomic groups. All known species and genotypes of *B. burgdorferi* sensu lato are tick transmitted and are maintained in nature by complex zoonotic transmission cycles, involving more than 50 avian and mammalian wildlife species as reservoir hosts for the spirochetes in Europe (3, 14).

Six genospecies, *B. afzelii*, *B. garinii*, *B. valaisiana*, *B. lusitaniae*, *B. bissettii*, and *B. burgdorferi* sensu stricto, are known to be prevalent in Europe (2, 4, 7, 28, 29). All these genospecies may cocirculate in local tick populations, suggesting that the local diversity of *B. burgdorferi* sensu lato can be as high as the regional or continental diversity (5, 7, 24, 26, 27, 29). Individual ticks may be infected with multiple genospecies of *B. burgdorferi* sensu lato; however, mixed infections seem to be much rarer than single infections (4, 7, 8, 11, 13, 16, 26, 27). Although information on the pattern of mixed infections in individual ticks may reveal important principles of the biology and ecology of *B. burgdorferi* sensu lato, only a few studies provide information on this aspect (4, 5, 7, 10, 11, 13, 16, 26).

Adult questing *Ixodes ricinus* ticks were collected in sylvatic habitats by blanket dragging (14) in the springs of 1996 and 1999. The habitats were located around Riga (Latvia), Bratislava (Slovakia), Bonn (Germany), and Lisbon (Portugal, two distinct sites) and in the New Forest (United Kingdom). *B. burgdorferi* sensu lato infection status was determined by PCR and the reverse line blot assay as described previously (2, 16, 23, 26).

The null hypothesis that the different genotypes were distributed independently of each other in individual ticks was statistically tested. The frequency data from each country were considered separately. The probability of a tick containing any one genotype was calculated from the data as follows: Σ(all ticks containing that genotype, whether as mixed or single infection)/Σ(all ticks tested). The probabilities of mixed and single infections were calculated assuming independent distributions in order to generate a set of expected frequencies.

A total of 1,483 questing adult ticks were analyzed, and 461 (31.0%) were found to be infected with *B. burgdorferi* sensu lato (Table 1). The most prevalent genospecies was *B. afzelii* (39.3%), followed by *B. garinii* (21.2%), *B. valaisiana* (12.8%), *B. lusitaniae* (5.8%), and *B. burgdorferi* sensu stricto (1.5%). The three genospecies *B. afzelii*, *B. garinii*, and *B. valaisiana* were present in all local tick populations collected in Slovakia (Bratislava), Latvia (Riga), Germany (Bonn), and Portugal (the Mafra site). These three genospecies accounted for 94.2% of all infections (Table 1; Fig. 1). Of the 410 typeable infections, 372 (90.7%) were single infections. Of the ticks infected with *B. garinii*, 24% were infected simultaneously with *B. valaisiana*, and 34% of the ticks harboring *B. valaisiana* were concurrently infected with *B. garinii* (Fig. 1). In contrast, only 1% of the *B. afzelii* infections occurred together with *B. garinii*, and no mixed infection of *B. afzelii* and *B. valaisiana* was observed. *B. afzelii* was absent from the United Kingdom site (16) and the Grândola site in Portugal (2). Therefore, these two sites were omitted in the statistical analysis. Of the remaining four sets of frequency data presented in the table, three have sufficient data to statistically test the hypothesis that the different genotypes are distributed independently of each other in individual ticks, namely, the data from Slovakia, Latvia, and Germany. Fewer mixed infections of *B. garinii-B. afzelii* composition were detected in individual ticks than expected in each of the three cases, and in two out of three this difference was significant. Significantly more mixed infections of *B. garinii-B. valaisiana* composition than expected under neutralist assumptions were observed in the data set from Slovakia (Slovakia, χ_2^2

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TABLE 1. Infection of questing adult *I. ricinus* with *B. burgdorferi* sensu lato[a]

Country or site	No. of ticks tested	No. (%) of ticks infected by different genospecies									
		BBU sensu lato	BBU	BGA	BVA	BAF	BLU	BGA/BVA	BGA/BAF	BBU/BAF	UT
Slovakia	585	237 (40.5)	3 (1.3)	37 (15.6)	22 (9.3)	125 (52.7)	0 (0)	20 (8.4)	2 (0.8)	2 (0.8)	26 (10.9)
Latvia	300	94 (31.3)	3 (3.1)	32 (34.0)	17 (18.0)	37 (39.4)	0 (0)	2 (2.1)	0 (0)	2 (2.1)	1 (1.0)
Germany	226	41 (18.1)	0 (0)	14 (34.0)	5 (12.2)	18 (43.9)	0 (0)	1 (2.4)	0 (0)	0 (0)	3 (7.3)
PM	217	32 (14.7)	0 (0)	8 (25.0)	12 (37.5)	1 (3.1)	0 (0)	6 (18.7)	0 (0)	0 (0)	5 (15.6)
Subtotal	1,328	404 (30.4)	6 (1.5)	91 (22.5)	56 (13.8)	181 (44.8)	0 (0)	29 (7.2)	2 (0.5)	4 (1.0)	35 (8.7)
PG[b]	55	41 (74.5)	0 (0)	0 (0)	0 (0)	0 (0)	27 (65.8)	0 (0)	0 (0)	0 (0)	14 (34.1)
United Kingdom[c]	100	16 (16.0)	1 (6.2)	7 (43.7)	3 (18.7)	0 (0)	0 (0)	3 (18.7)	0 (0)	0 (0)	2 (12.5)
Total	1,483	461 (31.0)	7 (1.5)	98 (21.2)	59 (12.8)	181 (39.3)	27 (5.8)	32 (6.9)	2 (0.4)	4 (0.9)	51 (11.1)

[a] BGA, *B. garinii*; BVA, *B. valaisiana*; BAF, *B. afzelii*; BLU, *B. lusitaniae*; UT, untypeable. PM, Portugal, Mafra site; PG, Portugal, Grândola site.
[b] Data published in reference 2.
[c] Data published in reference 16.

$= 96.1$, $P = 1.05 \times 10^{-20}$; Latvia, $\chi_1^2 = 6.41$, $P = 0.0113$; and Germany, $\chi_1^2 = 1.38$, $P = 0.240$).

The present study yielded two major findings: (i) the majority of infected ticks harbored one genospecies only, and (ii) *B. garinii* and *B. valaisiana* constituted the majority of multiple infections, whereas the combination of *B. garinii* and *B. afzelii* occurred significantly less frequently than expected.

It has previously been shown that the PCR targeting the 5S-23S intergenic spacer of *B. burgdorferi* sensu lato does not preferentially amplify *Borrelia* genotypes (2, 16, 26). In addition, genotyping of PCR products by the reverse line blot assay readily detects mixed infections at different ratios of DNA (16, 26), indicating that the present findings are likely to reflect the true genospecies diversity of *B. burgdorferi* sensu lato in the ticks analyzed. No DNA probe specific for *B. bissettii* was available. It is, therefore, possible that some of the untypeable samples fall into this genospecies.

It is now accepted that *B. afzelii*, *B. garinii*, and *B. valaisiana* are the most abundant genospecies in central Europe, often prevailing sympatrically in local *I. ricinus* populations (4, 7, 11, 16, 26, 27–29). The data from the present study are in line with these previous studies. As questing adult *I. ricinus* ticks have a history of two blood meals on diverse hosts (25), *Borrelia* infections may accumulate with the number of infectious blood meals (10, 12, 16). Cumulative acquisition of spirochetes upon consecutive infectious blood meals or superinfection of ticks already infected transovarially could explain the presence of dual infections in individual ticks. In addition, cotransmission of multiple strains from an individual host that contains a mixed infection may result in polyclonal infection of a single tick. Assuming equal transmission coefficients for the *Borrelia* genospecies throughout the transmission cycle (25), the frequency distribution of genotypes in individual ticks should match the frequency distribution at the population level. The pattern that emerges from the present study, however, indicates that this is not the case; *B. garinii* and *B. valaisiana* on the

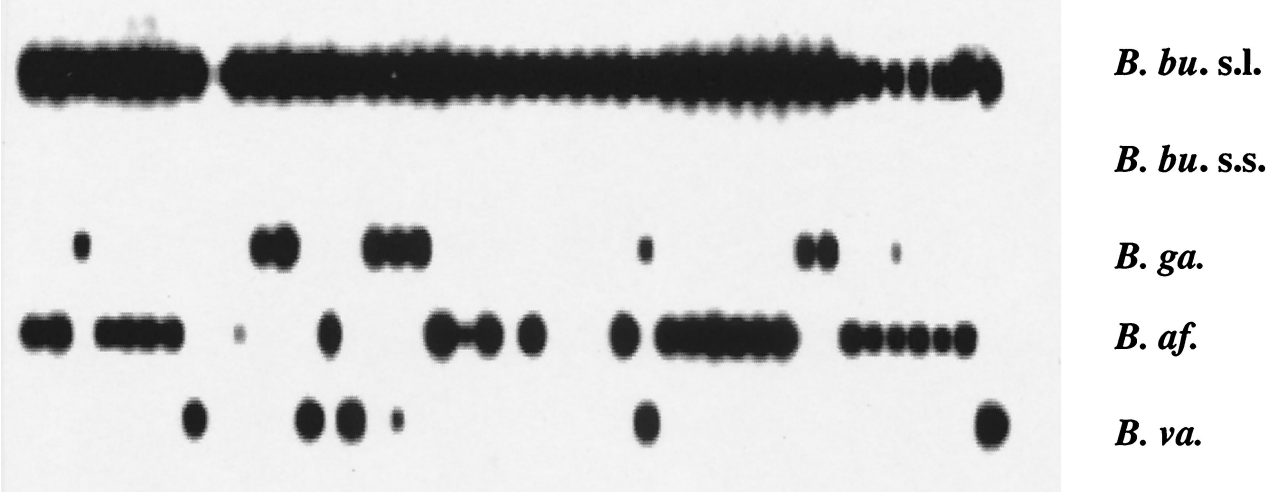

FIG. 1. Reverse line blot assay on PCR products of the 5S-23S intergenic spacer of *B. burgdorferi* sensu lato (*B. bu.* s.l.) derived from questing adult *I. ricinus* ticks from Slovakia. Representative samples are shown. Briefly, PCR products of *B. burgdorferi* sensu lato were hybridized to membrane-bound DNA probes specific for *B. burgdorferi* sensu lato, *B. burgdorferi* sensu stricto (s.s.), *B. garinii* (*B. ga.*), *B. afzelii* (*B. af.*), and *B. valaisiana* (*B. va.*). No DNA probe was available for *B. bissettii*. The PCR products per tick sample are ordered from the left to the right.

one hand and *B. azfelii* on the other hand seem to colonize individual ticks in a mutually exclusive way. This pattern cannot be attributed to geographic separation, since these three genospecies were found to circulate sympatrically in all of the central European sites analyzed. For this reason, it is likely that physiological or immunological factors shape the diversity of *B. burgdorferi* sensu lato in individual ticks.

In this paper an underlying mechanism is proposed for this observed pattern. It has recently been reported that the genospecies of *B. burgdorferi* sensu lato differ in their resistance to the alternative pathway of the hosts' complement system, depending on the genetic background of the spirochete and the source of serum (15). Rodent complement readily lyses particular genotypes of cultured *B. garinii* (type strains 20047, prevalent in western Europe) and *B. valaisiana* but not *B. afzelii*, whereas avian complement lyses the spirochetes in the reverse pattern. Interestingly, *B. burgdorferi* sensu stricto displays partial resistance to both avian and mammalian complement (15). The pattern of complement-mediated lysis matches the pattern of transmission competence of the major rodent and avian hosts for *B. burgdorferi* sensu lato (8–10, 16, 21), suggesting that resistance and/or sensitivity to complement is a key factor in Lyme disease ecology (15). The fact that *B. burgdorferi* sensu stricto exhibits only partial resistance to complement from mammalian and avian hosts from Europe may in part explain its low prevalence in the Old World.

Host complement has been demonstrated to be active in the midgut of feeding *I. ricinus* ticks (22). It is likely, therefore, that host complement selectively acts on spirochetes in the midgut of the tick with an effect similar to that observed in vitro, thereby effectively reducing the probability of avian- and rodent-associated *Borrelia* to coinfect individual ticks.

Although apparently rare, mixed infections of *B. garinii* and *B. afzelii* do occasionally occur in individual ticks (7, 8, 11, 13). This may be related to the presence of complement-deficient vertebrate hosts (1) or to the circulation of particular genotypes of *B. burgdorferi* sensu lato. In fact, diverse genotypes of *B. garinii* from localities in eastern Europe and Asia as well as OspA serotype 4 strains have been linked with rodent-tick transmission cycles (6, 18–20). In addition, it is possible that spirochetes evade complement-mediated selection in systemically infected ticks (17).

Altogether, the data corroborate earlier observations that the genotypes of *B. burgdorferi* sensu lato are not only differentially acquired from a host but are also differentially transmitted to the next developmental stage of the tick. In quantitative terms, host-to-tick and tick-to-tick transmission coefficients (25) seem to vary significantly with the genotype of *Borrelia* and source of blood meal. The findings of this study support the conclusion that *B. burgdorferi* sensu lato is maintained in nature through distinct transmission cycles, mainly involving small mammalian and avian hosts.

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