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Molecular Characterization of *Borrelia burgdorferi* sensu lato from Slovenia Revealing Significant Differences between Tick and Human Isolates

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One hundred twenty-nine Slovenian isolates of *Borrelia burgdorferi* sensu lato derived from patients (69 strains) or *Ixodes ricinus* ticks (60 strains) were characterized. All of the strains were first- or second-passage isolates obtained in 1992 and 1993 from the same endemic region. The techniques used for the molecular analysis of strains included species-specific polymerase chain reaction (PCR) typing, and pulsed-field gel electrophoretic separation of undigested and *Mlu*I-digested genomic DNA. Isolates were identified to the species level by large restriction fragment pattern (LRFP) analysis and the results compared with the species-specific PCR result. Fifty-two patient isolates (75%) were typed as *Borrelia afzelii* (LRFP MLa1), 6 (9%) as *Borrelia garinii* (LRFPs MLg1–4), and 11 (16%) as *Borrelia burgdorferi* sensu stricto. The latter included 9 isolates (13%) with a new LRFP that is not typical of *Borrelia burgdorferi* sensu stricto and for which the designation MLx is suggested. In contrast, only 32 of 60 (53%) tick isolates were typed as *Borrelia afzelii*, while 20 strains (33%) were typed as *Borrelia garinii* and 8 strains (13%) as *Borrelia burgdorferi* sensu stricto. Three new LRFPs were found among the *Borrelia garinii* (MLg5 and 6) and *Borrelia burgdorferi* sensu stricto (MLb15) tick isolates. Large restriction fragment pattern analysis identified new groups of *Borrelia burgdorferi* sensu lato and revealed an apparent difference in the isolation frequency of different species from patients and ticks in the same endemic region.

Lyme borreliosis is a multisystem infection originally considered to be caused by a single bacterial species, *Borrelia burgdorferi* (1, 2). Subsequently, in many separate studies of large numbers of individual isolates of the spirochaete conducted using a variety of molecular and genetic criteria (3–18), it was established that *Borrelia burgdorferi* comprised at least three distinct species (7). These were designated *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* (7), and *Borrelia afzelii* (19). Whereas all three species were found in Europe, only one species, *Borrelia burgdorferi* sensu stricto, was believed to be present on the North American continent. However, two genomic groups were recently identified among North American *Borrelia burgdorferi* sensu stricto isolates (20), and other *Borrelia* spp., isolated from *Ixodes* ticks in Japan and North America, have been described (21–23). These latter species have not as yet been shown to be associated with Lyme borreliosis.

The term *Borrelia burgdorferi* sensu lato (3) is currently used to describe patient and ixodid tick isolates of *Borrelia* spp. that have not been identified to species level.

Slovenia is a small, northern Adriatic country that is endemic for Lyme borreliosis and its tick vector, *Ixodes ricinus*. Recent estimates based on the culturing of ticks have shown that approximately 13% of nymphs and 27% of adult *Ixodes ricinus* throughout Slovenia are infected with *Borrelia burgdorferi* sensu lato, while in the Ljubljana re-

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Table 1: *Borrelia* species and strains shown in Figures 1 to 3

Species	Strain designation	Large restriction fragment pattern	Biological origin[a]
B. *burgdorferi* sensu stricto	297 (ATCC 53899)	MLb2	cerebrospinal fluid
B. *burgdorferi* sensu stricto	SL-6	MLb2	skin (EM)
B. *burgdorferi* sensu stricto	SL-16	MLb8	skin (EM)
B. *burgdorferi* sensu stricto	Ro-1	MLb8	tick[b]
B. *burgdorferi* sensu stricto	Ro-2	MLb15	tick
B. *burgdorferi* sensu stricto	Ro-6	MLb15	tick
B. *burgdorferi* sensu stricto	Ko-34	MLb15	tick
B. *burgdorferi* sensu stricto	Ko-40	MLb15	tick
B. garinii	20047	MLg1	tick
B. garinii	PBi	MLg2	cerebrospinal fluid
B. garinii	SL-9	MLg1	skin (EM)
B. garinii	SL-36	MLg2	skin (EM)
B. garinii	SL-56	MLg3	skin (EM)
B. garinii	SL-63	MLg4	skin (EM)
B. garinii	Ko-9	MLg2	tick
B. garinii	Ko-10	MLg2	tick
B. garinii	Ko-11	MLg2	tick
B. garinii	Ko-12	MLg2	tick
B. garinii	Ko-13	MLg5	tick
B. garinii	Ko-15	MLg3	tick
B. garinii	Ko-18	MLg2	tick
B. garinii	Ko-19	MLg4	tick
B. garinii	Ko-26	MLg1	tick
(*B. burgdorferi* sensu stricto)/*B. garinii*	Ko-28	MLg6	tick
B. garinii	Ko-33	MLg2	tick
B. afzelii	PGau	MLa1	skin (ACA)
B. afzelii	SL-3	MLa1	skin (ACA)
B. afzelii	SL-11	MLa1	skin (EM)
B. afzelii	SL-39	MLa1	skin (EM)
B. afzelii	SL-46	MLa1	skin (EM)
B. afzelii	SL-51	MLa1	skin (EM)
B. afzelii	SL-53	MLa1	skin (EM)
B. afzelii	SL-64	MLa1	skin (EM)
B. afzelii	SL-72	MLa1	skin (EM)
B. afzelii	Ko-14	MLa1	tick
B. afzelii	Ko-30	MLa1	tick
B. afzelii	Ko-41	MLa1	tick
B. afzelii	Rs-1	MLa1	tick
B. afzelii	Bl-5	MLa1	tick
B. burgdorferi sensu lato[c]	SL-73	MLX	skin (EM)
B. burgdorferi sensu lato[c]	SL-74	MLX	skin (EM *status post*)
B. burgdorferi sensu lato[c]	SL-75	MLX	skin (EM *status post*)
B. burgdorferi sensu lato[c]	SL-76	MLX	skin (EM)
B. burgdorferi sensu lato[c]	SL-78	MLX	lymphocytoma (breast)

[a]The geographic origin of all isolates was Slovenia with the exception of the following strains: 297 (ATCC 53899), USA; 20047, France; PBi, PGau, Germany.
[b]All tick isolates were derived from *Ixodes ricinus*.
[c]Isolates that were typed as *Borrelia burgdorferi* sensu stricto by species-specific PCR but possessed the atypical MLX LRFP.
ACA, acrodermatitis chronica atrophicans; EM, erythema migrans.

gion these figures rise to 21% and 30% respectively (24). We considered it of interest to perform a comparative molecular analysis of *Borrelia burgdorferi* sensu lato isolated in Slovenia from either patients or ticks over the same time period. We sought to compare the genetic and antigenic profiles of low-passage isolates with the aim of identifying differences between the two spirochaete populations and discovering correlations that might provide insight into the pathogenetic mechanisms of the disease process.

Since the elucidation of the diverse genetic nature of *Borrelia burgdorferi*, investigators have tried to find associations between the infecting species and the varied clinical manifestations of the disease. Some evidence for such associations has been uncovered (25–29). We also aimed to find further support for such relationships through our studies of patient isolates and considered that a detailed molecular characterization of strains might provide more information than species identification alone.

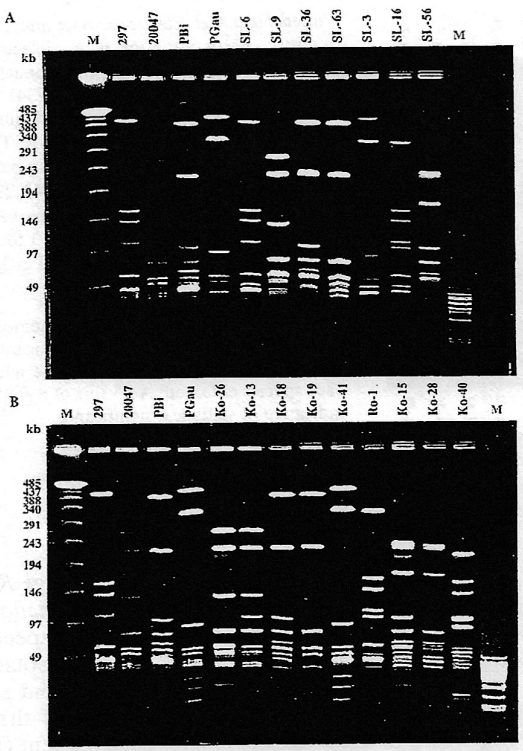

Figure 1: Pulsed-field gel electrophoretic separation of restriction enzyme *Mlu*I-digested genomic DNA from *B. burgdorferi* sensu lato isolates obtained from (A) patients, and (B) *Ixodes ricinus* ticks. Representatives of the different LRFPs encountered are presented in comparison to some reference strains. Strain designations are listed above their respective lanes. Lane M contains DNA molecular size markers [λDNA concatamers of 48.5–485 kb (first lane) or high molecular weight markers of 8.3–48.5 kb (last lane)].

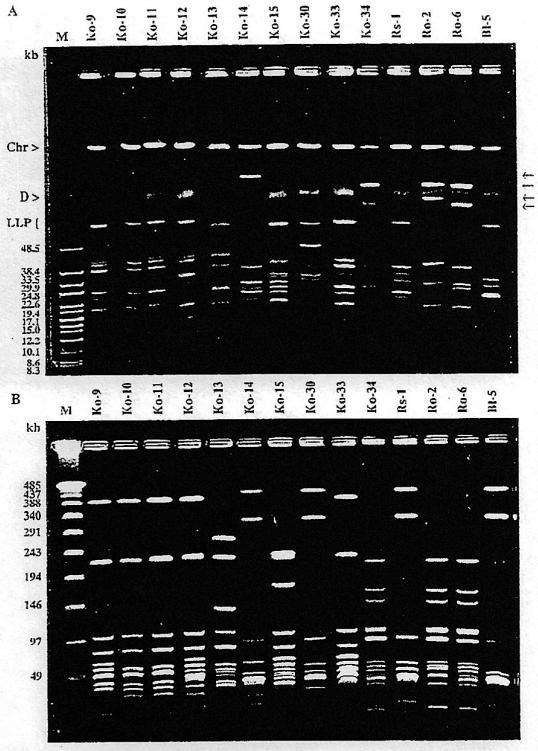

Figure 2: Pulsed-field gel electrophoresis of (A) undigested and (B) *Mlu*I-digested genomic DNA of *Borrelia burgdorferi* sensu lato isolates obtained from ticks. In three strains belonging to LRFP MLb15 (Ko-34, Ro-2, Ro-6) very large plasmids were encountered (1 large plasmid in Ko-34 and multiple large plasmids in strains Ro-2 and Ro-6). Strain designations are shown above their respective lanes. Lane M, DNA molecular size markers: high molecular weight markers of 8.3–48.5 kb (gel A) or λDNA concatamers of 48.5–485 kb (gel B). Chr >, the position of the 950 kb chromosome; ←, the position of the very high molecular weight plasmids; D >, diffuse band of varying intensity representing randomly sheared, chromosomal breakdown products; LLP, the position of the more usual 49–56 kb largest linear plasmids.

We therefore chose to study *Borrelia* spp. isolates using pulsed-field gel electrophoretic (PFGE) separation of (i) the genomic complement (plasmids and chromosome), and (ii) chromosomal DNA fragments generated by digestion of the genome with an infrequently cutting restriction endonuclease. Preliminary experiments with several such enzymes, identifiable from the restriction enzyme map of the *Borrelia burgdorferi* chromosome (30), showed that the enzyme *Mlu*I might be the most useful. In the course of this work, Belfaiza et al. (31) published similar studies using this enzyme and devised the term "large restriction fragment pattern" (LRFP) to describe the characteristic fragment patterns generated. For ease of comparison with their findings, we have retained their nomenclature. This study extends the catalogue of LRFPs associated with *Borrelia burgdorferi* sensu stricto and *Borrelia garinii*.

The aims of the study were (i) to assess differences, if any, in the molecular profiles (plasmid profiles and chromosomal restriction fragment patterns) of isolates derived from ticks and humans, and (ii) to look for associations between recognizable molecular characteristics of patient isolates (derived from the molecular profiling studies) and disease manifestation.

Materials and Methods

Bacterial Strains and Growth Conditions. The *Borrelia burgdorferi* sensu lato isolates shown in Figures 1 to 3 and men-

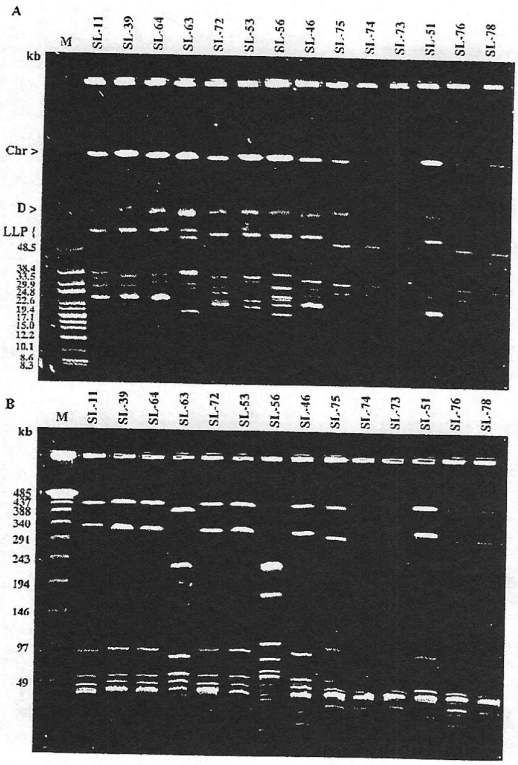

Figure 3: Pulsed-field gel electrophoresis of (A) undigested and (B) *Mlu*I-digested genomic DNA of *Borrelia burgdorferi* sensu lato isolates obtained from patients. For five strains belonging to LRFP MLx (SL-75, SL-74, SL-73, SL-76, SL-78) the size of the largest linear plasmids of these isolates is shown in contrast to those of *Borrelia afzelii* (MLa1) (strains SL-11, SL-39, SL-64, SL-72, SL-53, SL-46, SL-51) and *Borrelia garinii* [strains SL-63 (MLg4), SL-56 (MLg3)]. Strain designations are shown above their respective lanes. Lane M, DNA molecular size markers: high molecular weight markers of 8.3–48.5 Kb (gel A) or λDNA concatamers of 48.5–485 kb (gel B). Chr >, the position of the 950 kb chromosome; D >, diffuse band of varying intensity representing randomly sheared, chromosomal breakdown products; LLP, the position of the largest linear plasmids.

tioned in the text are listed in Table 1. Culture of patient and tick isolates was performed as described previously (24, 32). The reference strains, *Borrelia burgdorferi* sensu stricto strain 297 (ATCC 53899) and *Borrelia garinii* strain 20047, were obtained from Dr. Ira Schwartz, New York Medical College, Valhalla, New York, USA. The source of other reference strains has been described previously (11). A complete list of tick isolates, as well as the gender and stage of development of ticks from which the isolates were obtained, has been reported elsewhere (24).

Species Identification of Borrelia Isolates by Polymerase Chain Reaction. Polymerase chain reaction primers (33) designed to differentiate *Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* were used as described previously (34).

Pulsed-Field Gel Electrophoresis and Digestion of DNA in Agarose. Preparation of high molecular weight genomic DNA, PFGE, and restriction endonuclease digestion were performed as described previously (34). λDNA concatamers used as molecular size markers in Figures 1 to 3 were purchased from FMC Bioproducts, USA. The monomer size of these markers is 48.5 kb, resulting in bands of the following approximate sizes: 49, 97, 146, 194, 243, 291, 340, 388, 437, and 485 kb. The high molecular weight markers (Gibco-BRL Life Technologies, USA) used in Figures 1 to 3 produced bands of the following sizes: 8.3, 8.6, 10.1, 12.2, 15.0, 17.1, 19.4, 22.6, 24.8, 29.9, 33.5, 38.4, and 48.5 kb.

Statistical Analysis. The χ^2 test of homogeneity was used to determine whether there was a significant difference between patients and ticks with regard to the numbers of isolates of each species obtained. A p value of ≤ 0.01 was taken as being indicative of statistical significance.

Results

Identification of Distinct Large Restriction Fragment Patterns and Determination of Fragment Sizes. The results of species-specific PCR typing and LRFP analysis for the isolates used in this study are listed in Tables 1 and 2. Figures 1 to 3 present examples of each of the LRFPs found among the 69 Slovenian patient (Figures 1A and 3B) and 60 Slovenian tick (Figures 1B and 2B) isolates. Four reference strains (*Borrelia burgdorferi* sensu stricto strain 297, *Borrelia garinii* strains 20047 and PBi, and *Borrelia afzelii* strain PGau) were included in the gels of Figure 1 for comparison. The sizes of fragments obtained from *Mlu*I digestion of total genomic DNA for all the LRFPs encountered are presented in Table 3. Although we found the fragment patterns of established LRFPs to be the same as those published previously by Belfaiza et al. (31), our determinations of band sizes for certain fragments were different. Most of these differences were minor. However, we have previously commented upon one major difference associated with the LRFPs of *Borrelia burgdorferi* sensu stricto (34). This concerns the 145 kb band of MLbs 1–5, 8, and 10 that we calculated to be 160 kb. On the basis of the results of our PFGE analyses, we also believe that the largest fragment of LRFP MLg3 (220 kb) is a doublet. This is suggested by the PFGE-*Mlu*I profile of strain SL-56 (in Figure 1A) and strain Ko-15 (in Figure 2B). We measured the size of these two bands to be about 225 and 220 kb.

Among the patient isolates, strains with PFGE-*Mlu*I digestion profiles characteristic of the LRFPs MLb2 (strain SL-6), MLb8 (strain SL-16), MLg1 (strain SL-9), MLg2 (strain SL-36), MLg3

Table 2: Frequency of isolation of different large restriction fragment patterns (LRFPs) from patients and ticks.

Group	Species	LRFP	No. of patient isolates	No. of tick isolates
I	*B. afzelii*	MLa1	52	32
II	*B. garinii*	MLg5	0	1
		MLg1	1	2
		MLg2	2	12
		MLg4	2	2
		MLg3	1	1
		MLg6	0	2
III	*B. burgdorferi* sensu stricto	MLb2	1	0
		MLb8	1	2
		MLb15	0	6
IV	*B. burgdorferi* sensu lato*	MLX	9	0
Total			69	60

*Isolates that were typed as *B. burgdorferi* sensu stricto by species-specific polymerase chain reaction but possessed the atypical MLX LRFP.

(strain SL-56), MLg4 (strain SL-63), and MLa1 (strains SL-3, SL-11, SL-39, SL-46, SL-51, SL-53, SL-64, SL-72) are shown (Figures 1A and 3B). The tick isolates included representatives of LRFPs MLb8 (strain Ro-1), MLg1 (strain Ko-26), MLg2 (strains Ko-9, Ko-10, Ko-11, Ko-12, Ko-18, Ko-33), MLg3 (strain Ko-15), MLg4 (strain Ko-19), and MLa1 (strains Ko-14, Ko-30, Ko-41, Rs-1, Bl-5) (Figures 1B and 2B). In addition, four new LRFPs were encountered among the tick and patient isolates that have not previously been described.

Two new LRFPs were found among *Borrelia garinii* isolates derived from ticks. These are illustrated by the PFGE-*Mlu*I digestion profiles of strains Ko-13 and Ko-28 shown in Figures 1B and 2B. Like the other four MLg LRFPs described to date, they were characterized by fragments of 220 kb and 80 kb. We propose that these new LRFPs be designated MLg5 and MLg6, respectively. These LRFPs were very similar to the LRFPs MLg1 and MLg3. Thus, MLg1 differs from MLg5 by the lack of a single 100 kb band; MLg6 differs from MLg3 in the same manner (see Table 3). This also describes the relationship between MLg4 and MLg2. Thus, the six MLg LRFPs form three related pairs.

Another new LRFP was found among tick isolates typed as *Borrelia burgdorferi* sensu stricto using species-specific PCR. These strains also possessed the 140 kb fragment considered typical for the species (31). Three of these isolates are

Table 3: Sizes of bands obtained with *Mlu*I digestion of DNA from Slovenian *Borrelia burgdorferi* sensu lato isolates.

LRFP	Isolates shown in Figures 1 to 3	Presence of band with the following sizes (kb)														
		440	410	390	320	310	300	260	225	220	210	170	160	140	110	100	90	80
MLb2	297 [ATCC 53899], SL-6		+										+	+	+			
MLb8	SL-16, Ro-1					+							+	+	+	+		
MLb15	Ro-2, Ro-6, Ko-34, Ko-40										+		+	+		+	+	
MIx	SL-73, SL-74, SL-75, SL-76, SL-78	+					+									+		
MLg5	Ko-13							+		+				+		+		+
MLg1	20047, SL-9, Ko-26							+		+				+				+
MLg2	PBi, SL-36, Ko-9, Ko-10, Ko-11, Ko-12, Ko-18, Ko-33			+						+						+		+
MLg4	SL-63, Ko-19			+						+								+
MLg3	SL-56, Ko-15								+	+		+				+		+
MLg6	Ko-28								+	+		+						+
MLa1	PGau, SL-3, SL-11, SL-39, SL-46, SL-51, SL-53, SL-64, SL-72, Ko-14, Ko-30, Ko-41, Rs-1, Bl-5	+			+												+	

Table 4: Relationship between site of isolation and clinical symptoms for the groups of isolates (I-IV) based on genospecies and large restriction fragment pattern (LRFP).

Group no.	No. of isolates by site[a]					No. of patients with clinical symptoms			
	Skin				CSF	Local	Systemic		Other major manifestation[b]
	N	EM	LC	ACA			Mild	Severe	
I	1					0	0	1	0
		45[c]				24	21	7	1
				4		3	1	3	2
II		6				5	1	4	2
III		2				2	1	1	1
IV	2					1	1	1	2
		3				3	1	0	0
			1			1	1	0	0
					3	0	0	3	3

[a]N, normal appearing skin at the site of a previous tick bite and EM rash; EM, erythema migrans; LC, lymphocytoma; ACA, acrodermatitis chronica atrophicans; CSF, cerebrospinal fluid.
[b]Other major manifestation of Lyme borreliosis (36).
[c]Dual isolates were obtained from two patients with multiple EM. Only one isolate from each patient is considered for the purposes of this table.

shown in Figure 2B (Ro-2, Ro-6, and Ko-34) and one is shown in Figure 1B (Ko-40). The fragment sizes we measured for this LRFP were 210, 160, 140, 100, and 90 kb. This pattern of fragments does not resemble that of any of the 14 *Borrelia burgdorferi* sensu stricto LRFPs that have been described to date (31, 34). We therefore propose the designation MLb15 for this new LRFP.

Nine patient isolates were found to possess a new LRFP that we have previously encountered in the analysis of North American tick and mouse isolates from Millbrook, New York (strain 25015), and Cook County, Illinois (strain CT39) (34). The PFGE-*Mlu*I digestion profiles of five strains of this type (SL-73, SL-74, SL-75, SL-76, and SL-78) are shown in Figure 3B. All 11 strains (9 patient isolates, strain 25015 and strain CT39) were typed as *Borrelia burgdorferi* sensu stricto using species-specific PCR primers but possessed an atypical LRFP that lacked the 140 kb fragment thus far found in all isolates of *Borrelia burgdorferi* sensu stricto (31, 34). This LRFP is superficially very similar to the MLa1 LRFP of *Borrelia afzelii*. It is characterized by fragments of 440, 300, and 100 kb whereas the corresponding fragment sizes for the MLa1 LRFP are 440, 320, and 90 kb (see Table 3).

The 11 strains were also distinguishable from *Borrelia afzelii* by their possession of a large (presumably linear) plasmid that is considerably smaller in size than that found in *Borrelia afzelii* isolates (i.e. 56 kb; see Figure 3A) and more closely approximates the 49 kb linear plasmid found in *Borrelia burgdorferi* sensu stricto. Since the LRFP of these strains is not typical for *Borrelia burgdorferi* sensu stricto and it has been shown that strains 25015 and CT39 bear a peripheral relationship to strains of the latter species (17, 35), we have proposed that the LRFP be designated ML*x* until the taxonomic status of this group of isolates has been decided (34).

In two other instances, we also found a discrepancy between the results of species-specific PCR typing and LRFP analysis. One patient isolate was unusual in that it produced an amplified band of DNA with both the *Borrelia burgdorferi* sensu stricto and *Borrelia afzelii* PCR primers. However, the band produced with the latter primers was more intense than with the former. The LRFP of this isolate was clearly MLa1. Also, three strains isolated from ticks (1 MLg2 and 2 MLg6 isolates) produced bands with both the *Borrelia garinii* and *Borrelia burgdorferi* sensu stricto PCR primers. These latter isolates have been described in detail elsewhere (24).

Plasmids Associated with Borrelia burgdorferi sensu stricto MLb15. In addition to their unique LRFP, the six MLb15 isolates possessed another distinctive property. Figure 2 shows the PFGE profile of both undigested (Figure 2A) and *Mlu*I-digested (Figure 2B) genomic DNA from three of these isolates (Ko34, Ro2, Ro6). It is apparent from Figure 2A that instead of the typical 49 kb largest linear plasmid of *Borrelia burgdorferi* sensu stricto, these isolates contain two plasmids of undetermined structure and size (see Discussion) but of very high molecular mass. The possession of such plasmids was a feature shared by all six MLb15 strains. We encountered such large plasmids in only one other Slovenian strain: the

Borrelia afzelii (MLa1) tick isolate Ko-14 (shown in Figure 2A). None of the patient isolates was found to possess this characteristic.

Frequency of Isolation of Different Large Restriction Fragment Patterns from Patients and Ticks. On the basis of species-specific PCR typing and LRFP analysis we were able to classify the 69 patient and 60 tick isolates into four groups. The data are summarized in Table 2. Group I comprised strains typed as *Borrelia afzelii* using species-specific PCR primers and possessing the MLa1 LRFP that typifies this species (31). This group comprised 52 of 69 patient isolates (75%) and 32 of 60 tick isolates (53%). Group II comprised strains typed as *Borrelia garinii* using species-specific PCR primers and possessing one of the MLg LRFPs typical of this species (31). This group comprised 6 of 69 patient isolates (9%) and 20 of 60 tick isolates (33%). Among the tick isolates, we found strains with LRFPs (MLg5 and MLg6) that appeared to be variants of the two previously described LRFPs MLg1 and MLg3 (31). Group III comprised strains typed as *Borrelia burgdorferi* sensu stricto using species-specific primers and possessing one of the typical MLb LRFPs described for this species (31, 34). Two of 69 patient isolates (3%) (1 MLb2 and 1 MLb8) and 8 of 60 tick isolates (13%) (2 MLb8 and 6 MLb15) were included in this category. The MLb15 category alone comprised 10% of the tick isolates. Group IV comprised the nine patient isolates that typed as *Borrelia burgdorferi* sensu stricto using species-specific PCR primers but possessed the ML*x* LRFP (34). These strains comprised 9 of 69 patient isolates (13%). No strains of this type were encountered among our tick isolates from the same region.

Statistical Analysis. The numbers and percentages of the four groups of isolates obtained from patients and ticks may be summarized as follows: from patients, 52 *Borrelia afzelii* (75.4%), 6 *Borrelia garinii* (8.7%), 2 *Borrelia burgdorferi* sensu stricto (2.9%), 9 ML*x* (13%); from ticks, 32 *Borrelia afzelii* (53.3%), 20 *Borrelia garinii* (33.3%), 8 *Borrelia burgdorferi* sensu stricto (13.3%), 0 ML*x* (0%). When the χ^2 test of homogeneity was used to analyze these data, a significant difference ($p < 0.001$) was found between the numbers of isolates of each species obtained from ticks and patients.

Clinical Manifestations Associated with the Different Large Restriction Fragment Patterns. The clinical symptoms of patients associated with the four groups of isolates delineated by LRFP typing are summarized in Table 4. Local and systemic symptoms were present in 58% and 70% of patients, respectively. Local symptoms included itching, burning sensations, and/or pain; systemic symptoms included fatigue, headache, myalgias, arthralgias, and/or fever. Other major manifestations (36) of Lyme borreliosis were present in 16% of patients. These included meningoradiculitis (with or without peripheral facial palsy) and arthritis. The majority of patients had erythema migrans (EM) lesions. In three patients (1 from group I and 2 from group IV) isolates were obtained from biopsies of normal-appearing skin at the site of a previous tick bite and EM rash (32). Four patients had acrodermatitis chronica atrophicans. In three patients with central nervous system involvement, one patient had lymphocytic meningitis while two patients had neurologic involvement with CSF findings that were unremarkable.

Discussion

We characterized 129 isolates of *Borrelia burgdorferi* sensu lato derived from patients and the indigenous tick population in Slovenia, a region of Europe endemic for Lyme borreliosis. All of the strains were low-passage isolates, derived from the same, relatively small, geographic area (Slovenia is a small country of 20,251 square kilometers and approximately 2 million inhabitants) in 1992 and 1993. The study includes the largest collection of human isolates analyzed by PFGE to date. We have also extended the previous catalogue of LRFPs associated with *Borrelia garinii* (31) to 6 and *Borrelia burgdorferi* sensu stricto (31, 34) to 15 and identified a new group of patient isolates that are distinguishable on the basis of their characteristic ML*x* LRFP (34).

Some of our determinations of fragment sizes for the different LRFPs differ by small increments from those published previously (31). However, with few exceptions, the overall pattern of the fragments present in established LRFPs was the same as in the previous study (31). The major differences have been described in the Results section, and one of these (the 160 kb fragment of *Borrelia burgdorferi* sensu stricto) has been discussed previously (34). The precise molecular size of fragments remains to be determined.

Six isolates of *Borrelia burgdorferi* sensu stricto with the MLb15 LRFP were found among the tick

population but were not isolated from patients. This is suggestive of a lack of human pathogenic potential, but more strains of this type need to be isolated to support this hypothesis. Another interesting property of these strains was their plasmid content. We detected no apparent correlation between the plasmid content of strains and their LRFP type, except for these six MLb15 isolates. They lacked the typical 49 kb linear plasmid of *Borrelia burgdorferi* sensu stricto but possessed instead two plasmids of very high molecular mass. At present, it is not known whether these plasmids are linear or supercoiled, and the molecular size of plasmids cannot be assessed by comparison with linearized molecular size standards, regardless of their conformation (37). In pulsed-field gels they migrate with a relative mobility less than that of the 146 kb λ molecular size standard (unpublished data). We therefore conclude that they are 146 kb or more in size. We have also encountered very large plasmids of this type in a *Borrelia burgdorferi* sensu stricto tick isolate from Illinois, USA, which possessed another hitherto undescribed LRFP (MLb13) (34). However, other Illinois isolates with the same LRFP did not possess similar plasmids of high molecular mass.

The nine patient isolates which were typed as *Borrelia burgdorferi* sensu stricto by species-specific PCR but possessed the atypical ML*x* LRFP comprise the most interesting group of strains. On the basis of LRFP, protein, and plasmid profile analysis, they were found to resemble closely two North American isolates derived from an *Ixodes scapularis* tick (25015) and a white-footed mouse (CT39) (34). In addition to possessing the ML*x* LRFP, all 11 strains expressed major proteins that were indistinguishable in size from the OspA and OspB proteins of *Borrelia afzelii* [32 kDa and 35 kDa respectively (7, 25)] (data not shown). Also, all nine patient ML*x* isolates expressed a prominent protein that co-migrated with the 22 kDa proteins of strains 25015 and CT39 (data not shown). We have recently shown that the 22 kDa proteins of these latter strains are homologs of OspC (34). Discrepancies between species-specific PCR typing and LRFP analysis were also found in the case of four other strains. These isolates (1 patient isolate and 3 tick isolates) generated bands with more than one set of species-specific PCR primers. Possible explanations for these anomalous results have been discussed in detail elsewhere (24). However, it may be noted that our findings are compatible with sequence variation at the PCR-primer annealing site or mixed infections in which one species predominates. We have already noted one instance of mixed infection in ticks (34), and the simultaneous presence of different species in biological fluids from Lyme borreliosis patients has also been reported (38).

Several European study groups have reported correlations between the different clinical manifestations of Lyme borreliosis and the infecting species of *Borrelia burgdorferi* sensu lato (25–29). These studies suggest a predominant association of *Borrelia afzelii* with cutaneous manifestations of the disease in that the majority of isolates from erythema migrans and acrodermatitis chronica atrophicans lesions were found to be of this species. In contrast, isolates from more disseminated disease such as neuroborreliosis or arthritis were found to be *Borrelia garinii* or *Borrelia burgdorferi* sensu stricto. Our results are broadly in agreement with these previous findings for *Borrelia afzelii*. Thus, we found that 52 of 66 isolates from skin typed as *Borrelia afzelii*. Of the six *Borrelia garinii* isolates and two *Borrelia burgdorferi* sensu stricto isolates that we obtained, all were derived from erythema migrans lesions, and only two of the eight patients manifested extracutaneous symptoms associated with disseminated disease (one patient had meningoradiculoneuritis and the other arthritis). However, it should be pointed out that the majority of our isolates were from patients manifesting symptoms of "early" Lyme borreliosis; we do not know how many of these patients would have eventually gone on to develop severe disseminated disease if left untreated. Also, the association of *Borrelia afzelii* with cutaneous symptoms was not exclusive since at least one patient had a major neurologic manifestation of the disease and two of the four acrodermatitis chronica atrophicans patients had other major extracutaneous manifestations at the time of biopsy. Wilske et al. (25) have reported the isolation of three strains of *Borrelia afzelii* from CSF; presumably these patients had symptoms consistent with neuroborreliosis.

The most surprising finding of this study was the noticeable difference in the frequency of isolation of *Borrelia afzelii* and *Borrelia garinii* from patients and ticks. All the isolates used in this study were obtained from ticks collected in Slovenia or patients residing in Slovenia. None of the patients reported acquiring a tick bite outside of Slovenia. Thus, the tick isolates that we obtained were representative of the tick and spirochaete populations to which patients were exposed. Our results show

that the frequency of isolation of *Borrelia afzelii* appears to be over-represented in the patient population (75%) and under-represented in the tick population (53%). In contrast, *Borrelia garinii* appears to be over-represented among the ticks (33%) and under-represented among patients (9%). These differences were found to be statistically significant ($p < 0.001$). Moreover, the relative percentages of *Borrelia afzelii* and *Borrelia garinii* isolates present in ticks (53% and 33%, respectively) are derived from Slovenia as a whole (comprising 6 separate collection sites). At some of these collection sites the numbers of isolates obtained were very small and therefore presumably susceptible to a sampling bias. In the one area where *Borrelia burgdorferi* sensu lato was cultured from ticks in sizeable numbers, the ratio of *Borrelia afzelii* to *Borrelia garinii* isolates was more similar (43% and 40%, respectively) (24). Finally, the *Borrelia garinii* isolates were not only more numerous among the tick population than the human population, but also more varied. Thus, only MLg classes 1 to 4 were isolated from patients, whereas MLg classes 1 to 6 were found among ticks. Of the latter, MLg2 strains were the most numerous, comprising 12 (63%) of the 19 total tick isolates. Results with some similarities to the above have been reported previously (25). This study utilized an OspA serotyping scheme to classify and differentiate groups of tick and patient isolates. Comparison of the results from this study with our results is not straightforward since only three strains are common to both reports [*Borrelia burgdorferi* sensu stricto strain 297 (serotype 1), *Borrelia garinii* strain PBi (serotype 4), and *Borrelia afzelii* strain PGau (serotype 2)]. In agreement with our findings, the authors concluded that *Borrelia afzelii* (OspA serotype 2) was the most prevalent skin isolate among European strains. However, the interpretation of findings for *Borrelia garinii* is more difficult since this species was found to comprise five OspA serotypes (3 to 7) and the relationship of these five serotypes to our six MLg LRFPs is, at present, unknown.

The differences that we encountered in the rate of isolation of *Borrelia afzelii* and *Borrelia garinii* from ticks and humans are intriguing. Since *Borrelia garinii* of MLg classes 1 to 4 were found among the patient isolates, they are clearly able to cause Lyme borreliosis. However, it is not clear why an organism that is widespread within the tick population should be isolated so infrequently from patients and, therefore, apparently cause so few cases of disease. It could be argued that the spirochaetes isolated from patients represent the most rapidly growing variants of an initially mixed spirochaete population, thus biasing the results in favour of one species, i.e. *Borrelia afzelii*. However, our isolation rate from ticks did not suggest that *Borrelia garinii* spirochaetes have a lesser potential for growth in artificial media. We did observe gross differences in the growth rate of original isolates from both patients and ticks. However, the primary cultures used for the isolation of strains described in this study were incubated for three months before being discarded and, while some isolates were recovered only after such long incubation times, this did not correlate with species or tick versus human origin. We therefore do not believe that differences in growth rate between isolates can explain our results. Alternatively, it is possible that the findings reflect differences between *Borrelia afzelii* and *Borrelia garinii* in the number of organisms present in human skin (since the majority of our isolations were from cutaneous lesions). This could in turn affect the recovery rate and might also reflect the natural tropisms of the different species.

In conclusion, the principal finding of this study is an apparent difference in the distribution of *Borrelia burgdorferi* sensu lato species in patients and ticks. An association between infecting species and disease manifestations is less clear. We also conclude that LRFP analysis is a useful method of species identification that provides additional information concerning the genotypic characteristics of strains and permits the recognition of distinct subgroups within species.

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References

1. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP: Lyme disease – a tick-borne spirochetosis? Science 1982, 216: 1317–1319.
2. Johnson RC, Schmid GP, Hyde FW, Steigerwalt AG, Brenner DJ: *Borrelia burgdorferi* sp. nov.: etiological agent

of Lyme disease. International Journal of Systematic Bacteriology 1984, 34: 496–497.
3. Postic D, Edlinger C, Richaud C, Grimont F, Dufresene Y, Perolat P, Baranton G, Grimont PAD: Two genomic species in *Borrelia burgdorferi*. Research in Microbiology 1990, 141: 465–475.
4. Stälhammar-Carlemalm M, Jenny E, Gern L, Aeschlimann A, Meyer J: Plasmid analysis and restriction fragment length polymorphisms of chromosomal DNA allow a distinction between *Borrelia burgdorferi* isolates. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene 1990, 274: 28–39.
5. Rosa P, Hogan D, Schwan TG: Polymerase chain reaction identifies two distinct classes of *Borrelia burgdorferi*. Journal of Clinical Microbiology 1991, 29: 524–532.
6. Adam T, Graf B, Neubert U, Göbel UB: Detection and classification of *Borrelia burgdorferi* by direct sequencing of 16S rRNA amplified after reverse transcription. Medical Microbiology Letters 1992, 1: 120–126.
7. Baranton G, Postic D, Saint Girons I, Boerlin P, Piffaretti JC, Assous M, Grimont PAD: Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. International Journal of Systematic Bacteriology 1992, 42: 378–383.
8. Boerlin P, Péter O, Bretz AG, Postic D, Baranton G, Piffaretti JC: Population genetic analysis of *Borrelia burgdorferi* isolates by multilocus enzyme electrophoresis. Infection and Immunity 1992, 60: 1677–1683.
9. Marconi RT, Garon CF: Phylogenetic analysis of the genus *Borrelia*: a comparison of North American and European isolates of *Borrelia burgdorferi*. Journal of Bacteriology 1992, 174: 241–244.
10. Marconi RT, Garon CF: Identification of a third genomic group of *Borrelia burgdorferi* through signature nucleotide analysis and 16S rRNA sequence determination. Journal of General Microbiology 1992, 138: 533–536.
11. Picken RN: Polymerase chain reaction primers and probes derived from flagellin gene sequences for specific detection of the agents of Lyme disease and North American relapsing fever. Journal of Clinical Microbiology 1992, 30: 99–114.
12. Schwartz JJ, Gazumyan A, Schwartz I: rRNA gene organization in the Lyme disease spirochete, *Borrelia burgdorferi*. Journal of Bacteriology 1992, 174: 3757–3765.
13. Wallich R, Schaible UE, Lobet Y, Moter SE, Kramer MD, Simon MM: Evaluation of genetic divergence among *Borrelia burgdorferi* isolates by use of *ospA*, *fla*, HSP60, and HSP70 gene probes. Infection and Immunity 1992, 60: 4856–4866.
14. Welsh J, Pretzman C, Postic D, Saint Girons I, Baranton G, McClelland M: Genomic fingerprinting by arbitrarily primed polymerase chain reaction resolves *Borrelia burgdorferi* into three distinct phyletic groups. International Journal of Systematic Bacteriology 1992, 42: 370–377.
15. Ralph D, Postic D, Baranton G, Pretzman C, McClelland M: Species of *Borrelia* distinguished by restriction site polymorphisms in 16S rRNA genes. FEMS Microbiology Letters 1993, 111: 239–243.
16. Filipuzzi-Jenny E, Blot M, Schmid-Berger N, Meister-Turner J, Meyer J: Genetic diversity among *Borrelia burgdorferi* isolates: more than three genospecies? Research in Microbiology 1993, 144: 295–304.
17. Postic D, Assous MV, Grimont PAD, Baranton G: Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of *rrf*(5S)-*rrl*(23S) intergenic spacer amplicons. International Journal of Systematic Bacteriology 1994, 44: 743–752.
18. Liveris D, Gazumyan A, Schwartz I: Molecular typing of *Borrelia burgdorferi* sensu lato by PCR-restriction fragment length polymorphism analysis. Journal of Clinical Microbiology 1995, 33: 589–595.
19. Marin Canica M, Nato F, du Merle L, Mazie JC, Baranton G, Postic D: Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scandinavian Journal of Infectious Diseases 1993, 25: 441–448.
20. Assous MV, Postic D, Paul G, Nevot P, Baranton G: Individualisation of two new genomic groups among American *B. burgdorferi* sensu lato strains. FEMS Microbiology Letters 1994, 121: 93–98.
21. Kawabata H, Masuzawa T, Yanagihara Y: Genomic analysis of *Borrelia japonica* sp. nov. isolated from *Ixodes ovatus* in Japan. Microbiology and Immunology 1993, 37: 843–848.
22. Postic D, Belfaiza J, Isogai E, Saint Girons I, Grimont PAD, Baranton G: A new genomic species in *Borrelia burgdorferi* sensu lato isolated from Japanese ticks. Research in Microbiology 1993, 144: 467–473.
23. Marconi RT, Liveris D, Schwartz I: Identification of novel insertion elements, restriction fragment length polymorphism patterns, and discontinuous 23S rRNA in Lyme disease spirochetes: phylogenetic analyses of rRNA genes and their intergenic spacers in *Borrelia japonica* sp. nov. and genomic group 21038 (*Borrelia andersonii* sp. nov.) isolates. Journal of Clinical Microbiology 1995, 33: 2427–2434.
24. Strle F, Cheng Y, Nelson JA, Picken MM, Bouseman JK, Picken RN: Infection rate of *Ixodes ricinus* ticks with *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto. European Journal of Clinical Microbiology & Infectious Diseases 1995, 14: 994–1001.
25. Wilske B, Preac-Mursic V, Göbel UB, Graf B, Jauris S, Soutschek E, Schwab E, Zumstein G: An OspA serotyping system for *Borrelia burgdorferi* based on reactivity with monoclonal antibodies and OspA sequence analysis. Journal of Clinical Microbiology 1993, 31: 340–350.
26. van Dam AP, Kuiper H, Vos K, Widjojokusumo A, de Jongh MB, Spanjaard L, Ramselaar ACP, Kramer MD, Dankert J: Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. Clinical Infectious Diseases 1993, 17: 708–717.
27. Assous MV, Postic D, Paul G, Névot P, Baranton G: Western blot analysis of sera from Lyme borreliosis patients according to the genomic species of the *Borrelia* strains used as antigens. European Journal of Clinical Microbiology & Infectious Diseases 1993, 12: 261–268.
28. Anthonissen FM, De Kesel M, Hoet PP, Bigaignon GH: Evidence for the involvement of different genospecies of *Borrelia* in the clinical outcome of Lyme disease in Belgium. Research in Microbiology 1994, 145: 327–331.

29. Balmelli T, Piffaretti JC: Association between different clinical manifestations of Lyme disease and different species of *Borrelia burgdorferi* sensu lato. Research in Microbiology 1995, 146: 329–340.
30. Davidson B, MacDougall JH, Saint Girons I: Physical map of the linear chromosome of the bacterium *Borrelia burgdorferi* 212, the causative agent of Lyme disease. Journal of Bacteriology 1992, 174: 3766–3774.
31. Belfaiza J, Postic D, Bellenger E, Baranton G, Saint Girons I: Genomic fingerprinting of *Borrelia burgdorferi* sensu lato by pulsed-field gel electrophoresis. Journal of Clinical Microbiology 1993, 31: 2873–2877.
32. Strle F, Cheng Y, Cimperman J, Maraspin V, Lotric-Furlan S, Nelson JA, Picken MM, Ruzic-Sabljic E, Picken RN: Persistence of *Borrelia burgdorferi* sensu lato in resolved erythema migrans lesions. Clinical Infectious Diseases 1995, 21: 380–389.
33. Kuiper H, van Dam AP, Spanjaard L, de Jongh BM, Widjojokusumo A, Ramselaar TCP, Cairo I, Vos K, Dankert J: Isolation of *Borrelia burgdorferi* from biopsy specimens taken from healthy-looking skin of patients with Lyme borreliosis. Journal of Clinical Microbiology 1994, 32: 715–720.
34. Picken RN, Cheng Y, Han D, Nelson JA, Reddy AG, Hayden MK, Picken MM, Strle F, Bouseman JK, Trenholme GM: Genotypic and phenotypic characterization of *Borrelia burgdorferi* isolated from ticks and small animals in Illinois. Journal of Clinical Microbiology 1995, 33: 2304–2315.
35. Marconi RT, Konkel ME, Garon CF: Variability of *osp* genes and gene products among species of Lyme disease spirochetes. Infection and Immunity 1993, 61: 2611–2617.
36. Steere AC, Hutchinson GH, Rahn DWA, Sigal LH, Kraft GE, DeSanna ET, Malawista SE: Treatment of the early manifestations of Lyme disease. Annals of Internal Medicine 1983, 99: 22–26.
37. Norton Hughes CA, Kodner CB, Johnson RC: DNA analysis of *Borrelia burgdorferi* NCH-1, the first northcentral U.S. human Lyme disease isolate. Journal of Clinical Microbiology 1992, 30: 698–703.
38. Demaerschalck I, Messaoud AB, De Kesel M, Hoyois B, Lobet Y, Hoet P, Bigaignon G, Bollen A, Godfroid E: Simultaneous presence of different *Borrelia burgdorferi* genospecies in biological fluids of Lyme disease patients. Journal of Clinical Microbiology 1995, 33: 602–608.