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Infection Rate of *Ixodes ricinus* Ticks with *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* Sensu Stricto in Slovenia

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In spring 1993, *Ixodes ricinus* ticks were collected from six regions of Slovenia to determine their overall rate of infection with *Borrelia burgdorferi* sensu lato and to assess the frequency of individual species in these tick populations. Ticks were dissected and midgut tissue inoculated into modified Barbour-Stoenner-Kelly (BSK II) medium. *Borrelia* isolates were differentiated into separate species using species-specific polymerase chain reaction (PCR) primers and by large restriction fragment pattern (LRFP) analysis. Infected ticks were found in all six regions surveyed. Spirochaetes were isolated from 69 of 363 ticks (19 %): the isolation rate from adult female ticks was 35 % (23/66 ticks cultured), from adult male ticks 22 % (20/91), and from nymphal ticks 13 % (26/206). Determination of the species of 60 isolates revealed that 32 were *Borrelia afzelii* (53 %), 20 were *Borrelia garinii* (33 %), and 8 were *Borrelia burgdorferi* sensu stricto (13 %). In the Ljubljana region *Borrelia afzelii* and *Borrelia garinii* predominated (43 % and 40 %, respectively), whereas *Borrelia burgdorferi* sensu stricto constituted only 17 % of isolates. In three other regions of the country *Borrelia afzelii* was isolated exclusively, although the number of isolates investigated was small. This study demonstrates the presence of all three European species of *Borrelia burgdorferi* sensu lato within the Slovenian tick population and also within a geographic area of less than 100 m^2.

Lyme borreliosis is a widely distributed zoonosis caused by spirochaetes of the genus *Borrelia* (1, 2). In humans, the disease can affect a variety of organ systems including the skin, heart, joints, and nervous system (3). In Europe, at least three species are now known to be associated with the disease (*Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*) (4, 5); these three species are also known collectively as *Borrelia burgdorferi* sensu lato (6). Mammals (especially rodents) and birds can serve as natural reservoirs for *Borrelia burgdorferi* sensu lato, and ticks of the species *Ixodes* have been identified as the principal (and possibly exclusive) vector (2, 7). In Slovenia, the most abundant tick species is *Ixodes ricinus* (8). In 1988 and 1990 the rate of infection of this species with *Borrelia burgdorferi* sensu lato was determined to be 23 % for adult and 4 % for nymphal ticks (9, 10). However, these data were obtained using immunofluorescence assay (IFA) techniques that do not permit assessment of the frequency of infection with individual species.

Since 1986, Lyme borreliosis has been recognized as the most common tick-borne disease in Slovenia (11). From 1986 to the present, the number of patients diagnosed with Lyme borreliosis has risen annually (12). We considered the possibility that the observed changing epidemiology of human disease might be associated with an increased frequency of *Borrelia burgdorferi* sensu lato infection within the tick population. We therefore sought to assess the overall rate of infection of *Ixodes ricinus* in the same, small, well-defined geographic regions of Slovenia as had been investigated in earlier studies (9, 10). In addition, we wanted to document the presence and relative frequency of individual species of *Bor-*

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relia burgdorferi sensu lato within various geographic regions and thereby assess the potential contribution of each species to disease prevalence. For this reason we chose to use culture isolation of spirochaetes as the means of assessing infection rates, despite the fact that this makes comparison with previous results, derived from IFA investigations, more difficult.

Materials and Methods

Collection of Ticks and Isolation of Spirochaetes. The six regions selected for study are shown in Figure 1. Ticks were collected by flagging and dragging vegetation at the edge of forested areas; in the case of Koseze, the collection area was smaller than 100 m^2. Two areas (Koseze and Rakov Skocjan) were the sites of previous work (10); the four other regions (Kamniska Bistrica, Bohinjska Bistrica, Bled, and Roznik) were evaluated for the first time in the present study. Adult and nymphal ticks were collected, identified to the species level, and stored for further study at 4°C. Viable ticks were then immersed in 70 % ethanol for 2 to 3 min to reduce surface contamination, rinsed in sterile saline, and placed on new, glass microscope slides. Midguts from adult *Ixodes ricinus* were dissected out using new, sterile 18-gauge needles, and the midgut tissue was inoculated into Barbour-Stoenner-Kelly (BSK II) culture medium (13) modified by the addition of L cysteine (1 mM), dithiothreitol (1 mM) (14), rifampin (40 µg/ml), and ciprofloxacin (0.4 µg/ml) (15). Nymphal ticks were halved vertically with a new, sterile 18-gauge needle, and the entire tick was placed in culture medium. Cultures were incubated at 33°C and evaluated weekly for the presence of spirochaetes by darkfield microscopy. Negative cultures were discarded after eight weeks of incubation.

Species Identification of Borrelia Isolates by Species-Specific Polymerase Chain Reaction. Species-specific PCR primers designed to differentiate *Borrelia burgdorferi* sensu lato into the three separate species (*Borrelia burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*) were used in PCR amplification reactions as previously described (16).

Large Restriction Fragment Pattern Analysis. The species of the isolates was also determined using LRFP analysis (17). Pulsed-field gel electrophoretic (PFGE) separation of restriction enzyme *Mlu*I-digested genomic DNA was performed as previously described (18). The size of individual restriction enzyme fragments was determined using concatamers of bacteriophage λ (FMC Bioproducts, USA) as molecular weight standards. The designations of distinct *Mlu*I-PFGE profiles of isolates follows previously devised nomenclature (17).

Results

Table 1 shows the results of culturing ticks from each of the six areas studied. In total, 69 positive

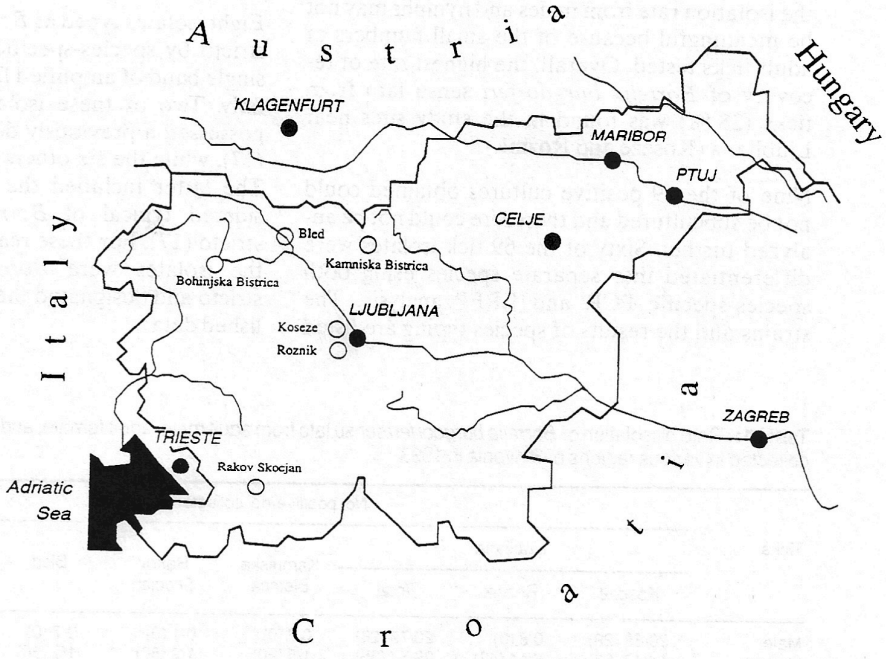

Figure 1: Map of Slovenia showing the location of sites surveyed. O = sites where *Ixodes ricinus* were collected.

cultures were obtained from the 363 ticks evaluated, yielding an overall infection rate of 19 %. In general, a higher rate of infection was found among adult ticks than among nymphs: 27 % of adult ticks were positive (43 of 157 ticks cultured) as opposed to 13 % of nymphs (26 of 206 ticks cultured). Among adult ticks, a higher rate of recovery of *Borrelia* isolates was obtained from females (35 % or 23 positives of 66 ticks cultured) than from males (22 % or 20 positives of 91 ticks cultured). In considering each of the six study sites individually, comparison of the isolation rate from adult and nymphal ticks was impaired by the small numbers of adult male and female isolates obtained from five of the six regions surveyed (Roznik, Kamniska Bistrica, Rakov Skocjan, and Bled, and Bohinjska Bistrica, where no adult male or female ticks were obtained). However, in the one region (Koseze) where adult male, adult female, and nymphal ticks were obtained in significant numbers, the isolation rate from females (33 %) slightly exceeded that from males (29 %) which, in turn, exceeded that from nymphs (19 %). In four of the other five regions (excluding Bohinjska Bistrica) the isolation rate from females was again the highest; however, in these regions a higher rate of isolation was obtained from nymphs than from males. This difference between Koseze and the other study sites in terms of the isolation rate from males and nymphs may not be meaningful because of the small numbers of adult ticks tested. Overall, the highest rate of recovery of *Borrelia burgdorferi* sensu lato from ticks (28 %) was found in the study sites near Ljubljana (Koseze and Roznik).

Nine of the 69 positive cultures obtained could not be subcultured and therefore could not be analyzed further. Sixty of the 69 tick isolates were differentiated into separate species using both species-specific PCR and LRFP analysis. The strains and the results of species typing are listed in Table 2. In general, there was good concordance between the two methods of species typing. Thus, all of the isolates that typed as *Borrelia afzelii* by species-specific PCR possessed the highly conserved MLa1 LRFP typical of this species (17). Among the *Borrelia garinii* isolates, we found strains representing the four MLg LRFPs described previously (17) and two new LRFPs that appeared to be closely related to MLg1 and MLg3; we have designated these MLg5 and MLg6 (unpublished data). As with the relationship between MLg2 and MLg4, the new MLg5 and MLg6 LRFPs differed from MLg1 and MLg3 in their possession of an extra 100 kb fragment (unpublished data). The six MLg LRFPs thus appear to comprise three related pairs and are therefore arranged as such in Table 2.

Three isolates (Ro-12, Ko-8, and Ko-28) produced an ambiguous species-specific PCR typing result: although a prominent band of the correct size was amplified by the *Borrelia garinii*–specific primers, a weak band was also produced with the *Borrelia burgdorferi* sensu stricto–specific primers. PFGE analysis produced a clear set of bands indicative of a single LRFP corresponding to MLg2 (Ro-12) or MLg6 (Ko-8 and Ko-28). No bands, either weak or prominent, corresponding in size to those of *Borrelia burgdorferi* sensu stricto LRFPs were present.

Eight isolates typed as *Borrelia burgdorferi* sensu stricto by species-specific PCR and produced a single band of amplified DNA with these primers only. Two of these isolates (Ro-1 and Ko-42) possessed a previously described LRFP (MLb8) (17), while the six others possessed a new LRFP. The latter included the 140 kb fragment considered typical of *Borrelia burgdorferi* sensu stricto (17). For these reasons we concluded that the isolates were *Borrelia burgdorferi* sensu stricto and designated the LRFP MLb15 (unpublished data).

Table 1: Rate of isolation of *Borrelia burgdorferi* sensu lato from adult male, adult female, and nymphal *Ixodes ricinus* ticks collected in various regions of Slovenia in 1993.

Ticks	No. positive/no. collected (% positive)							
	Ljubljana			Kamniska Bistrica	Rakov Skocjan	Bled	Bohinjska Bistrica	Total
	Koseze	Roznik	Total					
Male	20/68 (29)	0/8 (0)	20/76 (26)	0/7 (0)	0/1 (0)	0/7 (0)	0	20/91 (22)
Female	14/43 (33)	6/14 (43)	20/57 (35)	1/5 (20)	1/2 (50)	1/2 (50)	0	23/66 (35)
Nymph	7/37 (19)	3/10 (30)	10/47 (21)	2/40 (5)	3/15 (20)	6/79 (8)	5/25 (20)	26/206 (13)
Total	41/148 (28)	9/32 (28)	50/180 (28)	3/52 (6)	4/18 (22)	7/88 (8)	5/25 (20)	69/363 (19)

Table 2: *Borrelia* species obtained from *Ixodes ricinus* ticks in Slovenia[a]

Strain designation	Tick origin	Geographic origin	Species	LRFP
Group 1: *B. afzelii* isolates				
Ko-2, 3, 4, 7, 23, 29	Male	Ljubljana (Koseze)	*B. afzelii*	MLa1
Ko-5, 14, 22, 27, 30, 31, 35	Female	Ljubljana (Koseze)	*B. afzelii*	MLa1
Ko-37, 38, 41, 43	Nymph	Ljubljana (Koseze)	*B. afzelii*	MLa1
Ro-4, 5	Nymph	Ljubljana (Roznik)	*B. afzelii*	MLa1
Ro-11	Female	Ljubljana (Roznik)	*B. afzelii*	MLa1
Kb-5	Female	Kamniska Bistrica	*B. afzelii*	MLa1
Kb-8	Nymph	Kamniska Bistrica	*B. afzelii*	MLa1
Rs-1	Nymph	Rakov Skocjan	*B. afzelii*	MLa1
Bl-1, 3, 4, 5, 6, 7	Nymph	Bled	*B. afzelii*	MLa1
Bl-2	Female	Bled	*B. afzelii*	MLa1
Bb-1, 2	Nymph	Bohinjska Bistrica	*B. afzelii*	MLa1
Group 2: *B. garinii* isolates[b]				
Ko-13	Female	Ljubljana (Koseze)	*B. garinii*	MLg5
Ko-26, 36	Male	Ljubljana (Koseze)	*B. garinii*	MLg1
Ko-9, 10, 11, 16, 17, 18, 25, 39	Male	Ljubljana (Koseze)	*B. garinii*	MLg2
Ko-12, 33	Female	Ljubljana (Koseze)	*B. garinii*	MLg2
Ro-12	Nymph	Ljubljana (Roznik)	(*B. burgdorferi* sensu stricto)/*B. garinii*	MLg2
Rs-2	Female	Rakov Skocjan	*B. garinii*	MLg2
Ko-19	Nymph	Ljubljana (Koseze)	*B. garinii*	MLg4
Ko-32	Female	Ljubljana (Koseze)	*B. garinii*	MLg4
Ko-15	Male	Ljubljana (Koseze)	*B. garinii*	MLg3
Ko-8, 28	Female	Ljubljana (Koseze)	(*B. burgdorferi* sensu stricto)/*B. garinii*	MLg6
Group 3: *B. burgdorferi* sensu stricto isolates				
Ko-42	Nymph	Ljubljana (Koseze)	*B. burgdorferi* sensu stricto	MLb8
Ro-1	Female	Ljubljana (Roznik)	*B. burgdorferi* sensu stricto	MLb8
Ko-21, 40	Male	Ljubljana (Koseze)	*B. burgdorferi* sensu stricto	MLb15
Ko-34	Female	Ljubljana (Koseze)	*B. burgdorferi* sensu stricto	MLb15
Ro-2, 3, 6	Female	Ljubljana (Roznik)	*B. burgdorferi* sensu stricto	MLb15

[a] Nine of the positive tick cultures listed in Table 1 (1 adult male, 1 adult female, and 7 nymphs) did not survive and could not be speciated.
[b] The MLg LRFPs MLg5/MLg1, MLg2/MLg4, and MLg3/MLg6 form three pairs of related patterns.
LRFP: large restriction fragment pattern.

Table 3: Rate of isolation of different species of *Borrelia burgdorferi* sensu lato from *Ixodes ricinus* ticks collected in various regions of Slovenia in 1993.

Species	No. (%) isolated							
	Ljubljana			Kamniska Bistrica	Rakov Skocjan	Bled	Bohinjska Bistrica	Total
	Koseze	Roznik	Total					
B. afzelii	17 (44)	3 (38)	20 (43)	2 (100)	1 (50)	7 (100)	2 (100)	32 (53)
B. garinii	18 (46)	1 (13)	19 (40)	0 (0)	1 (50)	0 (0)	0 (0)	20 (33)
B. burgdorferi sensu stricto	4 (10)	4 (50)	8 (17)	0 (0)	0 (0)	0 (0)	0 (0)	8 (13)
Total*	39	8	47	2	2	7	2	60

* Nine of the positive tick cultures (1 adult male, 1 adult female, and 7 nymphs) did not survive and could not be speciated.

The total numbers of isolates of each species and from each of the six regions studied are shown in Table 3. Considering all six regions together, *Borrelia afzelii* isolates predominated, representing 32 of 60 isolates (53 %) identified to the species level. *Borrelia garinii* was found to be the second most common isolate, representing 20 of 60 isolates (33 %), and *Borrelia burgdorferi* sensu stricto was the least common isolate, representing 8 of 60 isolates (13%). However, in the study sites near Ljubljana (particularly Koseze) the numbers of *Borrelia afzelii* and *Borrelia garinii* isolates

Table 4: Rate of isolation of different species of *Borrelia burgdorferi* sensu lato from adult male, adult female, and nymphal *Ixodes ricinus* ticks collected in Slovenia in 1993.

Species	Males	Females	Nymphs	Total
B. afzelii	6 (32 %)	10 (45 %)	16 (84 %)	32 (53 %)
B. garinii	11 (58 %)	7 (32 %)	2 (11%)	20 (33 %)
B. burgdorferi sensu stricto	2 (11 %)	5 (23 %)	1 (5 %)	8 (13 %)
Total*	19	22	19	60

*Nine of the positive tick cultures (1 adult male, 1 adult female, and 7 nymphs) did not survive and could not be speciated.

were approximately equal, representing 43 % and 40 % of isolates, respectively. This contrasted with Kamniska Bistrica, Bohinjska Bistrica, and particularly Bled, where all of the isolates examined were *Borrelia afzelii*. Interpretation of the results from these regions was, however, impaired by the small numbers of total isolates obtained.

Table 4 presents the relative distribution of the three species of *Borrelia burgdorferi* sensu lato found among adult male, adult female, and nymphal ticks collected from the six regions. The results do not show any apparent correlation between borrelial species and the gender of ticks but may suggest an association with stage of development. Thus, 16 of 19 nymphs (84 %) investigated were found to be infected with *Borrelia afzelii* compared to 10 of 22 adult females (45 %) and 6 of 19 adult males (32 %).

Discussion

Over the past ten years, a number of reports have appeared on the prevalence of *Borrelia burgdorferi* sensu lato infection among *Ixodes ricinus* ticks in Europe (19–29); a few additional reports have dealt with the infection rate of *Ixodes hexagonus* (30) and *Ixodes persulcatus* (31). Recently, Gern et al. (32) summarized findings on the ecology of Lyme borreliosis in Europe. In countries where Lyme borreliosis was present, borreliae were usually found in ticks at every location examined. However, the rate of recovery of spirochaetes differed from region to region. On average, the infection rate of *Ixodes ricinus* larvae was found to be 1 to 3 % (25, 27, 29) while in nymphal and adult ticks the infection rate ranged from 7 to 43 % (19–29, 32). In some reports, the percentage of ticks infected with *Borrelia burgdorferi* sensu lato was higher in adult ticks than in nymphs (27, 28), but this was not a consistent finding (24, 26). The disparity could result from the different animal hosts serving as reservoirs of infection in different regions and their varying levels of competence for Lyme borreliosis spirochaetes (33). Alternatively, it could be a reflection of the sample bias inherent in considerations of small numbers. In agreement with some of the aforementioned studies, we found the infection rate among adult ticks (27 %) to be higher than that among nymphs (13 %). Also, in our study of 363 *Ixodes ricinus* ticks, the overall rate of infection with *Borrelia burgdorferi* sensu lato was found to be 19 %. However, comparison with the rates of infection found in other studies is difficult because of the disparities in the relative numbers of adult male, adult female, and nymphal ticks investigated.

In Slovenia, ticks of several different species have been described, including *Dermacentor marginatus*, *Rhipicephalus sanguineus*, *Hyaloma dromedari*, and *Ixodes ricinus* (8), with the latter being the most prevalent species. In previous studies on the prevalence of ticks infected with *Borrelia burgdorferi* sensu lato in Slovenia (9, 10), the ticks collected belonged exclusively to the species *Ixodes ricinus*. This was also true of the present study. Animal hosts for this tick species, which is found throughout the Slovenian region, have been described in detail elsewhere (34–36).

In a study conducted in 1990, Ruzic-Sabljic et al. (10) reported *Borrelia burgdorferi* sensu lato infection rates for *Ixodes ricinus* ticks collected from five different localities in the environs of Ljubljana, as well as from five other regions of Slovenia. In the present study, ticks were collected at two of these previous sites: one in the Ljubljana area (Koseze) and one at Rakov Skocjan. In the 1990 study, the infection rate of ticks was determined by an indirect immunofluorescent assay, while in the present report culture isolation of *Borrelia burgdorferi* sensu lato from ticks was used as the criterion for infection. It may be that the sensitivity of detection of indirect im-

munofluorescence is higher than the sensitivity of culture isolation (37), although it is not known by precisely how much. In 1990, 5 of 22 (32 %) adult ticks and 1 of 64 (2 %) nymphs collected from Koseze were positive for *Borrelia burgdorferi* sensu lato (10) while in 1993, 34 of 111 (31 %) adult ticks and 7 of 37 (19 %) nymphs were positive. These results demonstrate an apparently dramatic increase in the rate of infection among nymphs from 1990 to 1993 although, given the different methods used to assess infection rate in the two studies, the findings should be interpreted with caution. Comparison of the results for Rakov Skocjan shows that, in 1990, 0 of 2 adult ticks and 2 of 22 nymphs were positive for *Borrelia burgdorferi* sensu lato, while the corresponding values for 1993 were 1 of 3 for adult ticks and 3 of 15 for nymphs. In the additional four regions investigated in the present study, a relatively high percentage (20 %) of infected nymphs was found in Bohinjska Bistrica. However, the number of ticks examined was relatively small (0 males, 0 females, and 25 nymphs). In both the previous and present reports, the highest percentage of infected ticks was found in the Ljubljana area (10).

The isolation of all three species of *Borrelia* from Slovenian ticks was not unexpected since *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto have been shown to be present in many other European countries (4, 38, 39). However, the finding of all three species in the small, discrete, geographic area of Koseze (where the collection site was smaller than 100 m^2) was less predictable. In the Ljubljana region, *Borrelia afzelii* and *Borrelia garinii* were present in approximately equal numbers (43 % and 40 %, respectively), while *Borrelia burgdorferi* sensu stricto constituted only 17 % of isolates. Overall, *Borrelia afzelii* predominated (53 %) as a result of the contribution from the five other regions (although the numbers for these regions are small and the total may therefore reflect sampling bias). Similarly low numbers of *Borrelia burgdorferi* sensu stricto were found among our patient isolates from Slovenia (unpublished data) and have also been reported from other regions of Europe (39).

Three isolates (Ro-12, Ko-8, and Ko-28) produced an ambiguous PCR typing result: a strong band was produced with the *Borrelia garinii* primers and a weak band with the *Borrelia burgdorferi* sensu stricto primers. LRFP analysis showed a clear, unambiguous MLg LRFP. These findings could indicate sequence variation in the 16S rRNA sequences on which the species-specific primers are based. This might indicate the presence within our study population of genomic groups other than *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto. Recently, Postic et al. (40) described two such new genomic groups among European strains of *Borrelia burgdorferi* sensu lato (groups VS116 and PotiB2), defined on the basis of DNA-DNA reassociation studies and analysis of their *rrf* (5S)–*rrl* (23S) intergenic spacer regions. To date, no data on the LRFPs of these isolates have been presented. Alternatively, PCR analysis is capable of producing a result with very few spirochaetes as the starting material, whereas LRFP typing requires ~10^8 spirochaetes per agarose plug for bands of DNA to be visible. It is possible that these results represent mixed infection of ticks with *Borrelia garinii* and *Borrelia burgdorferi* sensu stricto in which the numbers of infecting *Borrelia garinii* organisms are present in the vast majority: that is, the relative intensity of the bands produced in PCR amplification reflects the relative numbers of each species present in the amplification target. This theory also requires that the two species should have equal growth rates and that their relative numbers are maintained during culture. In this case, the DNA fragments of the *Borrelia burgdorferi* sensu stricto LRFP would not be visible by ethidium bromide staining. It should be noted, however, that we have previously encountered one tick isolate that typed as *Borrelia garinii* by species-specific PCR (exhibiting no visible bands, either weak or strong, with other species primers) but which produced a clear, unambiguous MLa1 LRFP by PFGE analysis (unpublished data). We have also encountered tick isolates that exhibited LRFPs that were clearly a mixture of two distinct LRFP types (41).

In total, eight isolates of *Borrelia burgdorferi* sensu stricto were obtained, all of which came from the Ljubljana region. These isolates produced an amplified fragment of DNA with the *Borrelia burgdorferi* sensu stricto–specific PCR primers only, and in LRFP analyses, they all possessed the 140 kb fragment that typifies the species (17). However, six of these isolates possessed a new LRFP (MLb15) that we have thus far found only in ticks (unpublished data). These isolates were also characterized by another unique and distinctive feature in that they possessed two very large plasmids instead of the more usual largest linear plasmid of 49 kb (unpublished data). Currently, it is not known whether these large plasmids are linear or supercoiled.

In conclusion, we have confirmed the previous findings that *Borrelia burgdorferi* sensu lato is present in *Ixodes ricinus* ticks in a number of regions throughout Slovenia, and that the infection rate appears to be highest in the central (Ljubljana) region. Comparison of results from the present study with those obtained in 1990 suggest that the infection rate of nymphs in a small, geographically well-defined locality in a suburb of Ljubljana may have increased markedly over a three-year period. The present study has also established the presence of *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia burgdorferi* sensu stricto in Slovenia and in a geographic area smaller than 100 m^2.

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