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Density of Questing *Ixodes ricinus* Nymphs and Adults Infected by *Borrelia burgdorferi* Sensu Lato in Switzerland: Spatio-Temporal Pattern at a Regional Scale

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ABSTRACT

Lyme borreliosis, the most important vector-borne disease in the Northern hemisphere, causes health problem for populations in endemic areas. In the present study, the density of questing *Ixodes ricinus* ticks and their infection with *Borrelia burgdorferi* sensu lato (sl) was examined in 11 areas located on the Swiss Plateau and in an alpine valley. From 1999 to 2001, free-living *I. ricinus* ticks were collected on a monthly basis by flagging vegetation in these areas. Each tick was examined for the presence of *B. burgdorferi* sl using direct fluorescent antibody assay, and for isolation of the bacteria. *Borreliae* were characterized by PCR followed by RFLP. Density of questing ticks varied greatly between studied areas. *Borreliae* were observed in ticks collected in all investigated sites. However, the prevalence of infection differed significantly among areas. Infection prevalence varied from 9% to 40% in nymphs and from 22% to 47% in adults. Adult ticks were significantly more infected (129/366, 35%) than nymphs (109/552, 20%). There was no correlation between nymphal density and infection prevalence as well as between adult density and infection prevalence, but there was a correlation between density of ticks and density of infected ticks. During the spring peak of questing tick density, a range of 2–30.3 infected ticks per 100 m^2 was observed. *B. burgdorferi* sl isolates ($n = 129$) were obtained from ticks collected in 10/11 areas. Five *Borrelia* species were identifed: *B. garinii*, *B. burgdorferi* sensu stricto, *B. afzelii*, *B. valaisiana*, *B. lusitaniae*, and six mixed infections were also obtained. *Borrelia* species were heterogeneously distributed in the different areas.

Key words: Lyme borreliosis—*Ixodes ricinus*—Switzerland.

INTRODUCTION

LYME BORRELIOSIS, a multisystemic disease caused by *Borrelia burgdorferi* sensu lato (sl) (Burgdorfer et al. 1982), is an arthropod-borne disease distributed on the Northern hemisphere. *B. burgdorferi* sl spirochetes are mainly transmitted by some tick species of the *Ixodes ricinus* complex, and these ticks infest mammals, birds, and reptiles (Gern and Humair 2002).

Different species have been assigned to the *B. burgdorferi* sl group: *B. burgdorferi* sensu stricto (ss) (Johnson et al. 1984), *B. garinii* (Baranton et al. 1992), and *B. afzelii* (Canica et al. 1993). These three species are consistently associated with clinical manifestations in humans. In addition, other *Borrelia* species included in the *B. burgdorferi* sl. group—*B. valaisiana* (Wang et al. 1997), *B. lusitaniae* (Le Fleche et al. 1997), *B. bissettii* (Postic et al. 1998), *B. andersonii* (Marconi et al. 1995), *B. turdi* (Fukunaga et al. 1996), *B. tanukii* (Fukunaga et al. 1996), *B. japonica* (Marconi et al. 1995), and *B. sinica* (Masuzawa et al. 2001)—have been isolated from ticks or from their vertebrate hosts.

In Switzerland, various studies have de-

scribed the infection prevalence in *I. ricinus* ticks in locations situated North and South of the Alps (Aeschlimann et al. 1986, Miserez et al. 1990, Péter et al. 1995, Wicki et al. 2000, Bernasconi et al. 1997, Jouda et al. 2003, Jouda et al. in press); however, the density of ticks was investigated in only two of these studies (Jouda et al. in press). Since the risk for humans of acquiring an infection by a tick-borne pathogen may be related to the local density of infected vectors, we determined the extent of variation in questing tick density and their infection by *B. burgdorferi* sl in various geographic areas located on the Swiss Plateau and in an alpine valley over 3 years.

MATERIALS AND METHODS

Studied areas and tick collection

The study was carried out in the Western part of the Swiss Plateau (Switzerland), as well as in an alpine valley. We selected 11 areas (coordinates using the Swiss grid system CH-1903 as defined by the Swiss Topographic Office): Aigle (altitude 420 m, 563994/128790), Bavois (altitude 440 m, 531907/171132), Bière (altitude 800 m, 513845/1555263), Eclépens (altitude 485 m, 531336/168437), St-Livres (altitude 680 m, 517778/152534), Bois-de-Portes (altitude 480 m, 499894,130664), Bois-du-Château (altitude 475 m, 493108/118283), Brochus-Creuson (altitude 440 m, 498900/128450), Vaumarcus (altitude 480 m, 546669/190400), Viège (altitude 780 m, 634552/125852), and Interlaken (altitude 620 m, 63243/171149) (Fig. 1).

Host-seeking adult and nymphal ticks were collected on a monthly basis from March 1999 through November 2001, representing 28 sampling sessions. Questing ticks were collected by flagging vegetation using a 1-m^2 flag over a distance of 150 m. The flag was examined for ticks every 25 m. Questing tick density was expressed as the number of ticks per 100 m^2. An annual value for tick density called cumulated tick density (CTD) was obtained by integrating the linearly interpolated curve of the measured questing tick densities over 1 year (Eisen et al. 2003, Perret 2003, Jouda et al. in press). Nymphs and adults were treated separately. Peak tick density (PTD) was defined as the maximal tick density (Jouda et al. in press, Perret 2003).

Collected ticks were maintained in plastic vials containing a few blades of grass and transported to the laboratory where they were identified as species and developmental stages and analyzed for the presence of *Borrelia* infection.

Borrelia burgdorferi *infection in host-seeking ticks*

Each tick was cut into two pieces. One half was examined by Immunofluorescence (IF) us-

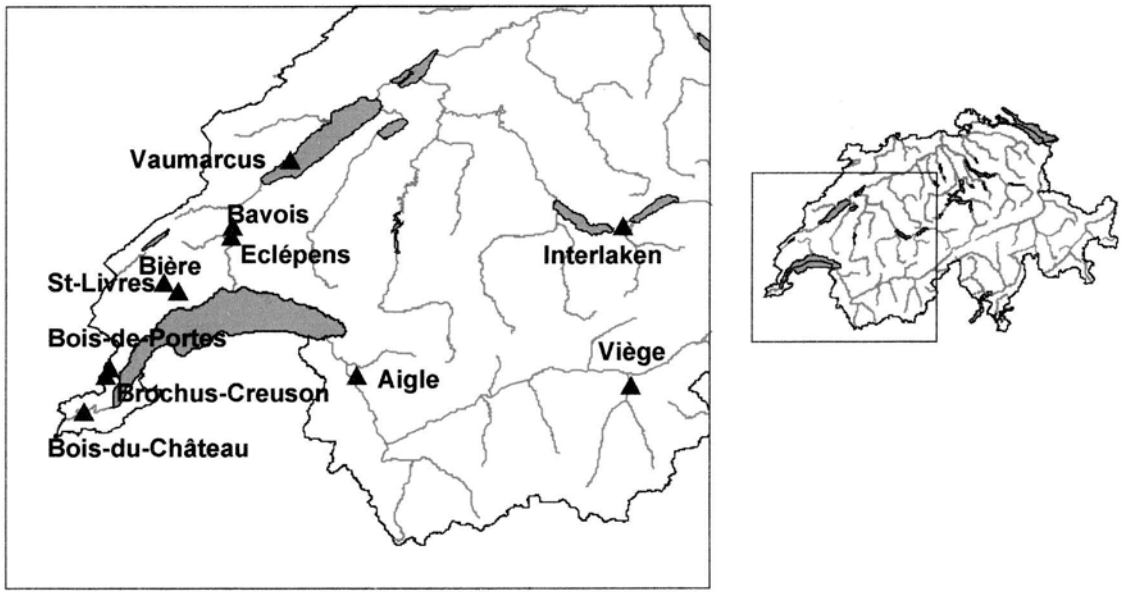

FIG. 1. Tick sampling areas in Western Switzerland (Support Cartographique OFS/OFT).

ing a fluorescein isothiocyanate-conjugated polyclonal antibody which was prepared from a pool of Lyme borreliosis patient sera and which detects all European *B. burgdorferi* sl species (Gern et al. 1999). Spirochete number was estimated, and infection degree was classified into three categories (Jouda et al. in press). Degree of infection in each tick was expressed as low, 1–50 spirochetes; medium, 50–500; and high, more than 500 spirochetes.

The other half of the tick used for *Borrelia* isolation was placed in culture tube containing BSK II medium (Sinsky and Piesman 1989), incubated at 34°C and examined by dark-field microscopy every 10 days for 2 months.

To evaluate the spatial variation in density of *I. ricinus* ticks infected with *B. burgdorferi* sl, we calculated the peak tick density of infected nymphs (PTDNi) and adults (PTDAi) by multiplying PTD by the percentage of infected ticks.

Identification of Borrelia species by PCR/RFLP

Isolates were typed with a restriction fragment length polymorphism on the PCR product according to Postic et al. (1994). Briefly, 1 mL of culture medium were centrifuged and washed two times, and then the pellet was suspended in 50 μL of ultrafiltered water. After incubation at 100°C for 10 min, the thermolysates were stored at −20°C until use for PCR reaction. Primers used to amplify the variable spacer region between two repeated genes encoding for ribosomal 23S and 5S were primer 1 (5′-CTGCGAGTTCGCGGGAGA-3′) and primer 2 (5′-TCCTAGGCATTCACCATA-3′). Each amplification reaction was carried out for 35 cycles. Denaturation was performed for 1 min at 94°C, annealing at 55°C for 1 min, and extension at 72°C for 1 min. PCR products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide. PCR products were first digested with *Mse*I restriction endonuclease for at least 2 h at 37°C, and then separated by electrophoresis in a 16% acrylamide gel for 1 h 30 min at 120 V. Digested DNA was stained with ethidium bromide.

Statistical analysis

Chi square tests, Fisher's exact test, correlation between tick density and infection prevalence, and correlation between tick density and density of infected ticks were calculated by using Splus version 6 for Windows (Insightful Corp., Seattle, WA). The correlations between CTD and year were calculated with R for Linux V 0.90.0.

RESULTS

Abundance of host-seeking ticks

Ticks belonging to *I. ricinus* only were collected by flagging vegetation at the different sampling areas. Altogether, 6768 *I. ricinus* ticks were collected: 1260 adults and 5508 nymphs. There was considerable variation in tick density among areas (Table 1). Tick density in Bois-du-Château was not recorded because this site was visited only three times during the study. The highest mean questing nymph and adult densities (CTD) for the three years were observed in St. Livres and Eclépens, respectively. In Vaumarcus, the forest at the study site was cut down during winter 1999–2000 to build a highway. This explains why the following year tick density, particularly density of nymphs, was drastically reduced. This site was the site with the highest PTD for nymphs in 1999, with 166 nymphs/100 m^2, and one with the lowest PTD in 2000, with 6 nymphs/100 m^2. Comparison of CTDs in nymphs among the 3 years showed that CTDs were the highest in 1999 in all sites and the lowest in 2000 in most sites (Table 1). In adults, highest CTDs were not related to specific year. Locations with high CTD in 1999 were also those with high CTD in 2000 and similarly between 2000 and 2001 (Fig. 2).

Borrelia burgdorferi sl in host-seeking ticks

The results on *Borrelia* infection in ticks are for years 2000 and 2001 except for Interlaken and Viège, where data from 1999 to 2001 are included. *Borrelia* infection analysis of ticks was performed on a randomly chosen number of ticks collected in each study site. Altogether, 918 ticks (552 nymphs and 366 adult ticks) were analyzed. *B. burgdorferi* sl infected ticks were recorded from all 11 sampled areas (Table 2). Overall, *B. burgdorferi* was infecting 109 (20%) of the 552 *I. ricinus* analyzed nymphs and 129 (35%) of the 366 adult ticks (Table 2). For all

OF *I. RICINUS* TICKS IN 10 SITES IN SWITZERLAND FROM 1999 TO 2001

	CTD[a]				*PTD*[b]			
	1999	*2000*	*2001*	*Mean*	*1999*	*2000*	*2001*	*Mean*
Nymphs								
Aigle	2193	804	2025	1674	17	7	21	15
Bavois	6861	1485	773	3040	85	11	9	35
Bière	1378	537	813	909	13	4	8	8
Bois-de-Portes	1550	455	331	779	17	3	4	8
Brochus Creuson	2174	2150	2722	2349	20	16	20	17
Eclépens	12113	4645	7168	7975	160	44	63	89
Interlaken	9432	2479	3764	5225	101	21	37	53
St. Livres	12152	10270	8667	10363	127	95	105	109
Vaumarcus	17880	531	nd	[c]	166	6	nd	[c]
Viège	4680	1190	2135	2668	43	18	17	26
Adults								
Aigle	564	204	293	354	6	2	2	3
Bavois	731	1172	522	808	7	11	5	8
Bière	54	214	358	209	1	2	2	<2
Bois-de-Portes	137	87	115	113	1	1	2	<2
Brochus Creuson	418	642	1141	734	3	5	10	6
Eclépens	2279	3041	2380	2567	18	23	17	19
Interlaken	791	1372	1539	1234	6	15	16	12
St. Livres	1277	2082	1859	1739	13	23	24	20
Vaumarcus	1943	1224	nd	[c]	19	18	nd	[c]
Viège	928	567	162	552	8	7	3	3

[a]Number of ticks per 100 m^2 and per year.
[b]Number of ticks per 100 m^2.
[c]Mean cannot be calculated because of destruction of biotopes in 2000.

studied areas grouped, the infection rate in adults was higher than in nymphs (Table 2) (Fischer's test, $p < 0.001$). The infection prevalences varied in nymphs from 9% in Viège to 40% in Bavois and from 22% in Viège to 47% in St. Livres in adult ticks (Table 2). Among studied areas, infection prevalences differed significantly (data from Bois-du-Château were not considered because this site was visited only three times during the study) (Chi-square test, $p = 0.0338$). Infection prevalences in nymphs and adults in the same site may be very similar like in Eclépens (34% vs. 35%) or very different like in St. Livres (14% vs. 47%), although tick densities in both areas are high.

During the spring peak of questing tick density, mean peak density (calculated for the 3 years) of infected ticks greatly varied among areas (Table 3). The lowest density reached 1.4 infected nymphs per 100 m^2 and 0.5 infected adults per 100 m^2 in Bière, whereas the highest densities were observed in Eclépens with 30.3 infected nymphs per 100 m^2 and in St. Livres with 7.4 infected adults per 100 m^2 (Table 3).

There was no correlation between nymphal density and infection prevalence (Spearman's rank correlation, $r^2 = 0.13982$, $p = 0.6882$) as well as between adult density and infection prevalence (Spearman's rank correlation, $r^2 = 0.1823$, $p = 0.5719$). However, there was a correlation between nymphal density and density of infected nymphs (Spearman's rank correlation, $r^2 = 0.9166$, $p = 0.0102$) and adult density and density of infected adults (Spearman's rank correlation, $r^2 = 0.9666$, $p = 0.0067$).

Spirochetes were isolated from 39 nymphs and 90 adults (35 males and 55 females) collected in 10 areas (no isolate was obtained from ticks collected in Bois-du-Château). Five *Borrelia* species were identified among isolates based on results of PCR/RFLP. *B. garinii* was the most frequently isolated species ($n = 59$) followed by *B. afzelii* ($n = 28$), *B. burgdorferi* ss ($n = 22$), *B. valaisiana* ($n = 12$), and *B. lusitaniae* ($n = 2$) (Table 2). In addition, five mixed infections

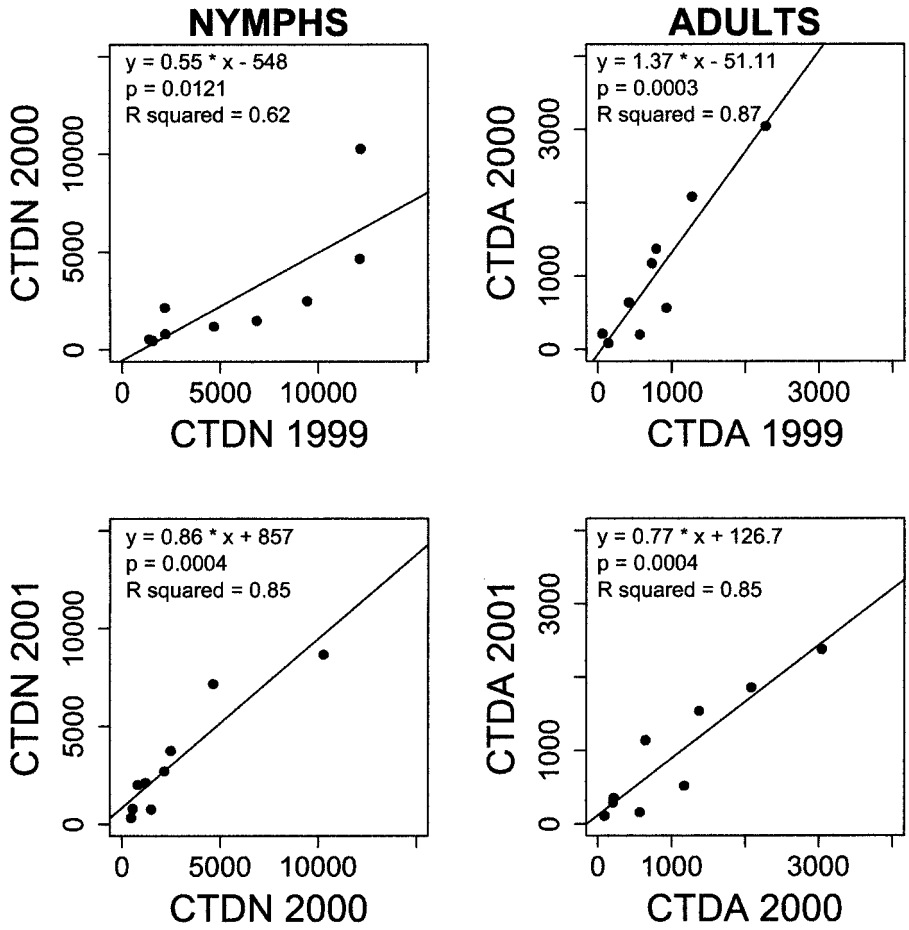

FIG. 2. Correlation of the cumulated tick density (CTD) between 1999 and 2000 (top row), 2000 and 2001 (bottom row) for nymphs (left column) and adults (right column). CTDN, cumulated nymph density; CTDA, cumulated adult density.

with two *Borrelia* species: *B. afzelii* and *B. garinii* ($n = 2$, from a nymph and an adult), *B. garinii* and *B. valaisiana* ($n = 2$, from female ticks), and *B. burgdorferi* ss and *B. afzelii* ($n = 1$, from a male tick) were obtained, as well as a mixed infection with three *Borrelia* species (*B. valaisiana*, *B. garinii*, and *B. lusitaniae*) from a male tick. *B. lusitaniae* was observed in three ticks all collected in the same site (St. Livres).

Spirochetes were counted in ticks, and we observed that 75% (82/109) and 77% (99/129) of infected nymphs and infected adults, respectively, were infected by less than 50 spirochetes. We observed that *B. afzelii*, *B. garinii*, and *B. valaisiana* spirochetes were often present in high numbers in ticks, whereas *B. burgdorferi* ss spirochetes usually infect ticks with a low load of spirochetes (Fig. 3). Interestingly, ticks presenting mixed infections by two or three *Borrelia* species ($n = 6$) were often (4/6) infected by less than 50 spirochetes.

DISCUSSION

Studies that address the epidemiology of human exposure to Lyme borreliosis must address the ecology and temporal distribution of nymphs and females of *I. ricinus*, the developmental stages that most often bite humans. Measurement of seasonal and annual changes in *I. ricinus* density with respect to the annual prevalence of *B. burgdorferi* sl in *I. ricinus* ticks is epidemiologically important. Therefore, we investigated *I. ricinus* density and the prevalence of *B. burgdorferi* sl in various areas in Switzerland over 3 years. Our data show that *I. ricinus* is the most common exophilic tick in

TABLE 2. *BORRELIA* INFECTION RATES OF *I. RICINUS* TICKS COLLECTED BY FLAGGING VEGETATION AT 11 SITES

	Infection rate[a]		*Isolates*						
Areas	*Nymphs*	*Adults*	*Ba*	*Bg*	*Bb*	*Bv*	*BI*	*Mixed*	*Total*
Aigle	16/47 (34%)	6/13 (46%)	0	4	2	3	0	0	9
Bavois	6/15 (40%)	9/20 (45%)	0	0	0	1	0	0	1
Bière	4/24 (17%)	3/11 (27%)	0	3	2	0	0	0	5
Bois-de-Portes	1/10	1/2	0	0	1	1	0	0	2
Bois-du-Château	2/11	0/4	0	0	0	0	0	0	0
Brochus Creuson	6/31 (19%)	6/25 (24%)	2	3	0	1	0	0	6
Eclépens	15/44 (34%)	24/69 (35%)	7	12	4	1	0	2	26
Interlaken	24/95 (25%)	41/112 (37%)	11	18	9	2	0	1	41
St. Livres	15/108 (14%)	15/32 (47%)	3	12	2	2	2	1	22
Vaumarcus	11/69 (16%)	15/37 (41%)	3	6	0	1	0	2	12
Viège	9/98 (9%)	9/41 (22%)	2	1	2	0	0	0	5
Total	109/552 (20%)	129/366 (35%)	28	59	22	12	2	6	129

[a]Number of ticks infected/number of ticks examined (%).
Ba, *B. afzellii*; Bg, *B. garinii;* Bb, *B. burgdorferi* ss; Bv, *B. valaisiana;* Bl, *B. lusitaniae.*

this country and confirm that *B. burgdorferi* sl is widespread (Aeschlimann et al. 1986, Miserez et al. 1990, Péter et al. 1995, Wicki et al. 2000, Bernasconi et al. 1997, Jouda et al. in press). In fact, all tick populations examined for *Borrelia* infection were infected. Infection prevalences varied from 9% to 40% in nymphs and from 22% to 47% in adults. Viège is the site showing the lowest infection prevalences (9% for nymphs and 22% for adults). This site is the only one located in the Alps. In most areas, the infection prevalences are among the highest recorded across Europe (Gray et al. 1998, Hubalek and Halouzka, 1998a). Infection prevalences in adults and in nymphs were not significantly related to tick densities. This is not surprising since it has been shown that within endemic areas in Europe, over the usual nymph density (range of 4–100 nymphs 100 m^2), which is the case in our study sites—there is no significant correlation between prevalences and nymph densities (Randolph 2001).

Mean infection prevalences recorded for *I. ricinus* among the various sites showed a 4.4-fold difference in nymphs and 2.1-fold difference in adults. The higher prevalence of *B. burgdorferi* infection in adults compared to nymphs has to be related to the higher number of bloodmeals ingested by adults. However, differences in infection prevalences between nymphs and adults in tick populations with similar densities were observed in Eclépens and St. Livres. This indicates that larval and nymphal hosts contribute differentially to *Borrelia* infection in ticks. Different species composition of mammal hosts in these areas such as the relative proportions of reservoir-competent and incompetent hosts, in the two areas or climatic conditions forcing larvae and nymphal ticks to feed on similar hosts (Randolph and Storey 1999) may lead to this situation. In Viège, very low infection rate in nymphs has been observed. This site as mentioned before is located in the Alps and in this area biotopes for reservoir hosts like *Apodemus* sp and *Clethrionomys* sp on which larval ticks abundantly feed, are less frequent (Hausser 1995). It has been suggested that low prevalence of spirochetal infection in *I. ricinus* nymphs results from lar-

TABLE 3. PEAK TICK DENSITY OF *I. RICINUS* NYMPHS AND ADULTS INFECTED BY *B. BURGDORFERI* SL IN DIFFERENT SAMPLING AREAS ON THE SWISS PLATEAU AND ALPINE AREA

Area	*PTDNi*[a]	*PTDAi*[b]
Aigle	5.1	1.5
Bavois	14.0	3.5
Bière	1.4	0.5
Brochus Creuson	3.5	1.4
Eclépens	30.3	6.8
Interlaken	13.3	4.6
St. Livres	15.3	7.4
Viège	2.3	1.3

[a]Infected nymphs/100 m^2.
[b]Infected adults/100 m^2.
PTDNi, peak tick density of *I. ricinus*–infected nymphs; PTDAi, peak tick density of *I. ricinus*–infected adults.

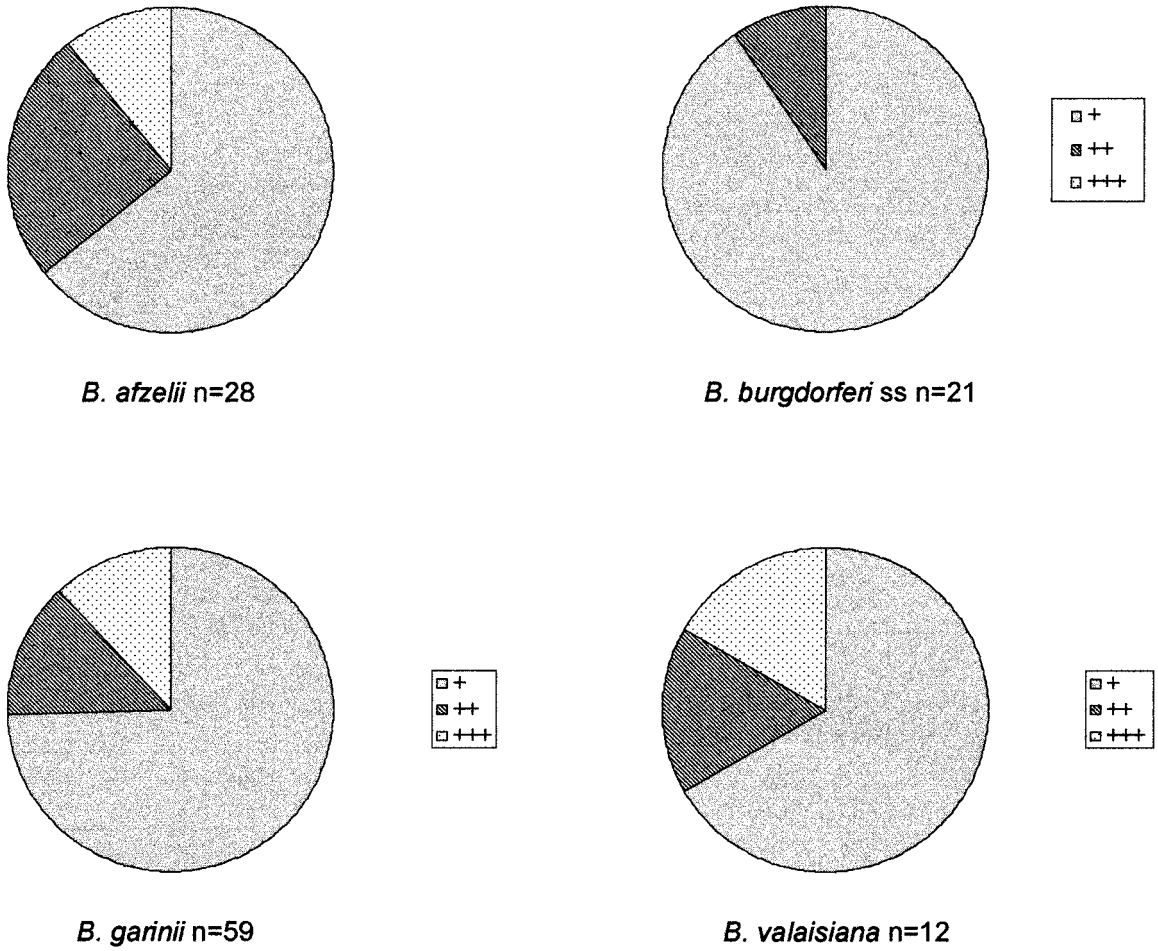

FIG. 3. Relation between *Borrelia* species and infection degree in *I. ricinus* ticks collected in the 11 sites.

val ticks feeding abundantly on reservoir-incompetent hosts such as deer (Jaenson and Tälleklint 1992, Matuschka et al. 1993, Gray et al. 1992, 1995, Tälleklint and Jaenson 1996). This might explain the lower *Borrelia* prevalence observed in Viège.

Studies on spatial variation in density of *I. ricinus* ticks infected with *B. burgdorferi* sl are rather rare. In southern part of Sweden, densities reaching 7.68 infected *I. ricinus* nymphs per 100 m^2 (Mejlon and Jaenson 1993, Tälleklint and Jaenson 1996) have been reported. In North America, densities of infected nymphs reached 0.77 infected *I. scapularis* nymphs per 100 m^2 in Connecticut (Stafford et al. 1998), 11.6 infected *I. scapularis* nymphs per 100 m^2 in New York (Daniels et al. 1998), and 22.17 infected *I. pacificus* nymphs per 100 m^2 in California (Tälleklint-Eisen and Lane 1999). In our study, these densities were higher reaching 30.3 infected nymphs (range 1.4–30.3 infected nymphs per 100 m^2) and 7.4 infected adults per 100 m^2 (range 0.5–7.4 infected adults per 100 m^2). However, we have to note that our data represent the maximum risk reached in the areas, when questing tick densities are the highest. Our data also showed that the risk to encounter an infected tick greatly varies among areas and even between very close sites.

Our results document the presence of *B. lusitaniae* in an additional location in Switzerland (Jouda et al. 2003, Jouda et al. in press). *B. lusitaniae* has been first isolated from *I. ricinus* ticks in Portugal (Nuncio et al. 1993) and has then been reported in the Czech Republic, Moldavia, Ukraine (Postic et al. 1997), Slovakia (Gern et al. 1999), Tunisia (Zhioua et al. 1999), Poland (Mizak et al. 2000), Spain (Escudero et al. 2000, Barral et al. 2002), France (Richter et al. 2003), and Switzerland (South of the Alps (Jouda

et al. 2003) and North of the Alps (Jouda et al. in press and present data). *B. lusitaniae* is very frequent in *I. ricinus* ticks in Portugal (De Michelis et al. 2000), in Tunisia (Younsi et al. 2001), and in Morocco (Sarih et al. 2003) and is only sporadically reported in other areas. Interestingly, in the present study, *B. lusitaniae* was isolated from three ticks, all collected at St. Livres. Reservoir hosts for all *Borrelia* species infecting *I. ricinus* have been identified except for *B. lusitaniae* (Gern and Humair 2002). The presence of *B. lusitaniae* associated with two *Borrelia* species (*B. garinii* and *B. valaisiana*) usually associated with birds (Gern and Humair 2002) is striking.

One of the main criticism of culturing *Borrelia* from ticks or vertebrate hosts is that culturing may select *Borrelia* in culture medium. The fact that mixed infections represented 5% of all obtained isolates and that 67% of them were obtained from ticks with a low load of spirochetes show that isolation of mixed infection is nevertheless possible. Higher mixed infection rates are usually detected in studies where *Borrelia* DNA is detected in ticks. However, PCR has been found to amplify DNA fragments of nonviable organisms (Varde et al. 1999), whereas by culturing we show that mixtures of various *Borrelia* species can survive in tick environment as well as in BSK medium.

The intensity of spirochetal infection in ticks may allow one to forecast the annual incidence of Lyme borreliosis in an area, as recently described by Hubalek et al. (2003). These authors reported a good correlation between annual incidence of Lyme borreliosis and ticks infected by >50 borreliae. Our survey confirms that most *I. ricinus* ticks are infected with a low load of spirochetes (Hubalek et al. 1998b, Gern et al. 1999, Hubalek et al. 2003). Interestingly, we observed that *B. burgdorferi* ss spirochetes usually infect *I. ricinus* with a low load of spirochetes; whether this is linked with the fact that *I. ricinus* is not a good vector for *B. burgdorferi* ss compared to *B. afzelii* as shown recently (Crippa et al. 2002) remains open.

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