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INFECTIOUS DISEASES

Distribution and prevalence of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks of canton Ticino (Switzerland)

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Abstract. Free-living *Ixodes ricinus* ticks were collected from 12 different sites of canton Ticino, south of the Alps (Switzerland). Each tick was examined for the presence of *Borrelia burgdorferi* sensu lato (sl), the etiologic agent of Lyme borreliosis using direct fluorescent antibody assay, and isolation of the bacteria. *Borreliae* were characterized by PCR followed by RFLP. The abundance and infection rates of *I. ricinus* ticks varied greatly between the areas. Two localities were found free of *Borrelia*. The prevalence of infected ticks ranged from 5 to 19%. Most ticks (96%) were found infected by < 50 spirochetes. Three *B. burgdorferi* sl species were successfully isolated: *B. garinii* dominated, followed by *B. lusitaniae* and *B. valaisiana*. Additionally, a mixed infection with *B. garinii* and *B. valaisiana* was observed. The distribution of the various *Borrelia* species in the different areas was heterogeneous. This is the first report of the presence of *B. lusitaniae* in *I. ricinus* in Switzerland.

Key words: *Borrelia burgdorferi* sensu lato, *Borrelia lusitaniae*, *Ixodes ricinus*, Switzerland

Introduction

In Europe, the etiologic agent of Lyme borreliosis, *Borrelia burgdorferi* sensu lato (sl) is transmitted by the bite of *Ixodes ricinus* ticks [1]. Five different *Borrelia* species have been found associated with *I. ricinus: B. garinii* [2], *B. burgdorferi* [2], *B. afzelii* [3], *B. valaisiana* [4] and *B. lusitaniae* [5]. In Switzerland, the infection rate of *I. ricinus* ticks collected in various areas varied between 5 and 47.5% [6]. South of the Alps, in canton Ticino, various studies have demonstrated the presence of *B. burgdorferi* sl in *I. ricinus* ticks [7]. However, no *Borrelia* isolate has been obtained till now from ticks collected in this part of Switzerland and only *B. valaisiana* DNA was detected by PCR [8] in ticks.

In the present study, we investigated the prevalence of *Borrelia* infection in *I. ricinus* ticks collected from vegetation and determined the presence and the geographic distribution of the various *Borrelia* species in different areas of Ticino.

Material and methods

Collection of ticks

During 3 years from 1999 to 2001, late May or early June, free–living ticks were collected by flagging a 1 m^2 cotton cloth through the vegetation at 12 localities in canton Ticino (Switzerland) (Figure 1). The number of ticks attached to cloth was counted every 18 m, and ticks were maintained in tubes containing grass until species determination and examination for *Borrelia* infection.

Borrelia *infection and isolation*

Each *I. ricinus* tick was dropped in 70% ethanol and was cut into two pieces, one half was examined by direct Immunofluorescence (IF) and the other half was incubated in BSKII medium modified according to Sinsky and Piesman [9] for *B. burgdorferi* sl isolation as described previously [10].

For IF, one half of the tick was spread on a glass slide, dried overnight at 37 °C and fixed in acetone for 10 min. Fluorescein isothiocyanate-conjugated antibodies prepared from a pool of Lyme borreliosis patient sera which detect all *Borrelia* species were used [11]. Slides were incubated in a humid chamber for 30 min at 37 °C. They were examined for *Borrelia* by fluorescence microscopy. Spirochete numbers were estimated as described by Gern et al. [10] and the degree of infection was expressed as low: 1–50 spirochetes, medium: 50–500 spirochetes, and high: more than 500 spirochetes.

The other half of the tick was individually transferred into culture tubes containing BSKII medium [9, 10] and was examined by dark-field microscopy for *Borrelia* after 1 week of incubation at 34 °C and every week for 1 month.

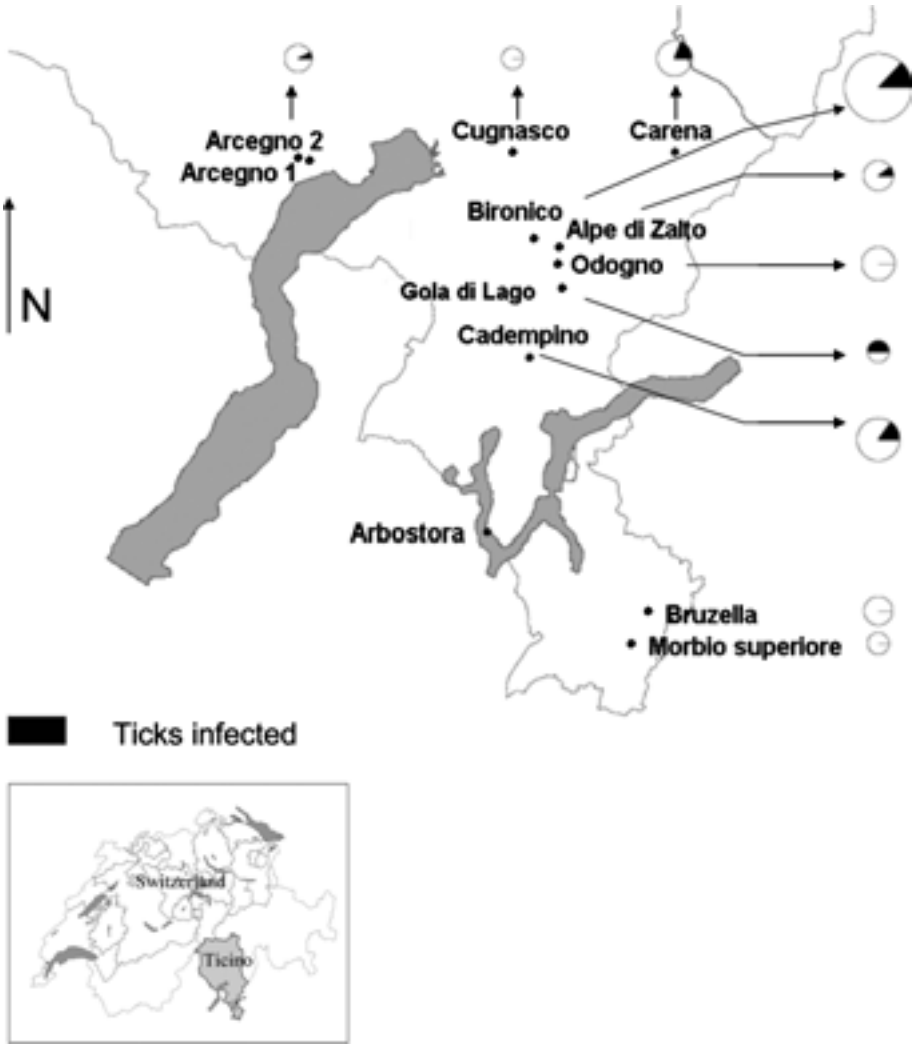

Figure 1. Distribution of *I. ricinus* infected by *Borrelia burgdorferi* sl in Ticino. The graphics (Sectors) represent the infection rate by *Borrelia burgdorferi* sl according to density of *I. ricinus* ticks collected in different sites of study (Direct immunofluorescence).

PCR and RFLP

Identification of *Borrelia* species infecting *I. ricinus* ticks was done by PCR followed by RFLP [12]. Primers were selected to amplify the variable spacer region between the 3′ end of the 5S rRNA (*rrf*) and the 5′ end of the 23S rRNA (*rrl*). PCR products were digested by *Mse* I endonuclease. In 1999 and 2001, all initial culture tubes containing ticks were examined by PCR and RFLP. In 2000, *Borrelia* DNA detection by PCR was made only in initial culture tubes containing ticks found infected by IF, and PCR/RFLP was used for characterization of *Borrelia* isolates.

Statistical analysis

Fisher's exact test for count data and χ^2 tests were calculated using R version 0.90.0 [12] on a Linux 2.2.5 computer. Correlation between tick infection rate and the altitude was calculated using SPSS version 10.00.

Results

Altogether, 460 ticks were collected from the study sites. All collected ticks belonged to the species *I. ricinus*. No tick was found at two out of 12 sites (Arcegno 1 and Arbostora): nymphs were collected in 10 sites whereas adults were collected in 7/10 sites (Figure 1). Tick density varied between 0 and 66 ticks/100 m^2 (Table 1). The highest densities were observed in Bironico and Cadempino (Figure 1). The density of collected nymphs did not differ in 1999, 2000 and 2001, the same observation was made for adult ticks, except for females collected in 2000, when only one female was collected (data not shown). A total of 460 *I. ricinus* ticks, 415 nymphs, 17 females and 28 males, were examined for *Borrelia* infection. The overall prevalence of *B. burgdorferi* sl in ticks by IF was 11% (50/460) (Table 2). A higher infection rate occurred in females (24%, 4/17) followed by males (11%, 3/28) and nymphs (10%, 43/415) (Table 2). However, there was no significant difference

Table 1. Tick densities in study sites in canton Ticino

Sites	Total length of drag (m^2)	Total number of ticks collected	Tick density (no. of ticks/ 100 m^2)
Bironico	378	249	66
Cadempino	324	66	20
Odogno	306	22	1
Gola di Lago	360	34	9
Alpe di Zalto	558	31	6
Morbio Superiore	360	1	<1
Bruzella	504	24	5
Arcegno1	514	0	0
Arcegno 2	450	15	4
Arbostora	414	0	0
Carena	144	16	11
Cugnasco	432	2	<1
Total	4744	460	10

between the prevalence in nymphs and adults ($p = 0.3103$), and between females and males ($p = 0.3986$). The highest infection prevalence in nymphs was obtained in 1999 with 14% (22/165) whereas in 2000 and 2001 the observed prevalences in nymphs were 7% (7/103) and 10% (14/143), respectively (Table 2). However, these differences were not significant ($p = 0.25362$, χ^2).

Borrelia infected nymphs were collected only in 6/10 localities where nymphs were collected, with an infection prevalence varying between 5% (1/22) and 19% (3/16) (detailed data not shown). Infected adults were found in only 2/7 sites (Bironico and Cadempino) where adults were collected, with an infection rate of 29% (4/14) and 19% (3/16), respectively. In 2000, none of seven adults examined revealed any *Borrelia* infection.

As mentioned before, each tick was examined for *Borrelia* infection using IF and isolation. A total of 460 ticks were examined and 57 (12%) were found infected by IF and/or isolation (Table 3). Among them 4/57 (7%) by IF and isolation, 46/57 (81%) were observed infected by IF only and 7/57 (12%) by isolation only.

A total of 11 *Borrelia* isolates were obtained from the 460 (2.4%) ticks placed in culture tubes (Table 3). These isolates were characterized by PCR/RFLP: they belonged to *B. garinii* (n = 7), *B. lusitaniae* (n = 1), *B. valaisiana* (n = 1), and 2 could not be characterized.

Table 3. Detection of *Borrelia burgdorferi* sl in *I. ricinus* ticks using IF and culture

	IF+/ Culture+	IF+/ Culture−	IF−/ Culture+	IF−/ Culture−
1999	2	23	4	157
2000	0	7	2	101
2001	2	16	1	145
Total	4	46	7	403

In addition, *Borrelia* DNA could be detected in eight tubes containing ticks found negative in culture and collected in 1999 and 2001: five of them were positive only by IF and three were positive only by detection of DNA by PCR. In 2000, *Borrelia* DNA detection by PCR was done only in culture tubes containing ticks found infected by IF (n = 7) and DNA was detected in 1/7. Among these nine positive PCR, RFLP allowed to identify DNA of *B. garinii* (n = 5), *B. lusitaniae* (n = 2) and *B. valaisiana* (n = 1) and one mixed infection with *B. garinii* and *B. valaisiana*.

Spirochete numbers were estimated in all 460 ticks examined by IF: 48/50 (96%) were infected by < 50 spirochetes and two (4%) by more than 500 spirochetes. Four *Borrelia* isolates were obtained from ticks found infected by < 50 spirochetes and *Borrelia* DNA was detected by PCR in six culture tubes containing ticks infected by < 50 spirochetes. All other isolates were obtained from ticks negative in IF.

Borrelia characterization by RFLP was possible in 20 ticks (11 from *Borrelia* isolates and 9 from *Borrelia* DNA detected in culture medium): *B. garinii* was the most frequent identified species with 65% (13/20) followed by *B. lusitaniae* (15%, 3/20) and *B. valaisiana* (10%, 2/20). A mixed infection with *B. garinii* and *B. valaisiana* was observed in one nymph (5%, 1/20). Nymphs were mainly infected by *B. garinii* (63%, 10/16), followed by *B. lusitaniae* (13%, 2/16) and *B. valaisiana* and a mixed infection (6%, 1/16). In females (n = 3), all three genospecies were present. The only isolate obtained from males was *B. garinii*. The highest variety of *Borrelia* species was observed in Bironico (*B. garinii*: 6/10, *B. lusitaniae*: 3/10, and *B. valaisiana*: 1/10), and in Cadempino (*B. garinii*: 2/5 (40%), *B. valaisiana*: 1/5 (20%) and one unidentified). In Odogno, *B. garinii* (n = 1) and one unidentified *Borrelia* were observed. In all other localities only

Table 2. *Borrelia* infection in *I. ricinus* ticks in Ticino (using direct immunofluorescence)

	1999	2000	2001	Total
Tick stadium	Nb inf/examined	Nb inf/examined	Nb inf/examined	Nb inf/examined
Nymphs	22/169 (14%)	7/103 (7%)	14/143 (10%)	43/415 (10%)
Females	1/8 (13%)	0/1	3/8 (38%)	4/17 (24%)
Males	2/9 (22%)	0/6	1/13 (19%)	3/28 (11%)
Adults	3/17 (18%)	0/7	4/26 (15%)	7/45 (16%)

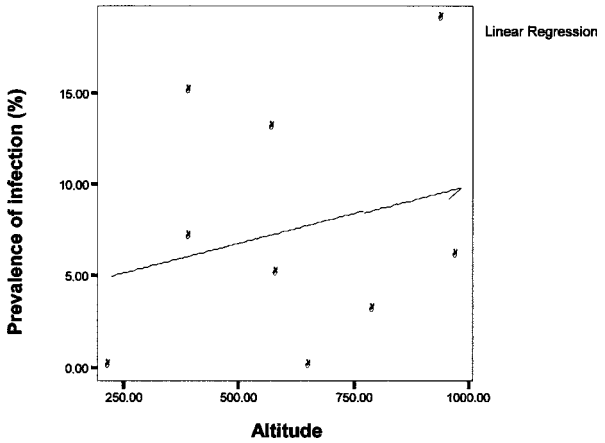

Figure 2. Prevalence of *Borrelia* infection in *I. ricinus* ticks in relation to the altitude (using Immunofluorescence).

B. garinii was present in ticks: two ticks were infected by *B. garinii* in Alpe di Zalto and one in Gola di Lago.

Since collection site altitude varied from 224 to 980 m, we examined whether there was a relationship between infection in ticks and altitude. The data showed that the prevalence of infection in ticks was not correlated by altitude (Figure 2).

Discussion

Various studies have been realized in canton Ticino, south of the Alps, in Switzerland on ticks and tick-borne pathogens [7]. Different tick species have been described in this canton, among them *I. ricinus*. In our study, only *I. ricinus* ticks were collected by flagging vegetation in the studied areas.

The density of questing ticks collected varied greatly between the different areas. The highest density of ticks was observed in Bironico with 66 ticks/100 m^2 (62 nymphs/100 m^2 and 4 adults/100 m^2). Miserez et al. [7] also observed the highest number of *I. ricinus* ticks in Bironico, whereas no tick was collected in Odogno and Morbio Superiore. In our study, *I. ricinus* ticks were collected in these two localities. A study realized in Europe by Gray et al. [13] on tick densities showed that most studied areas displayed similar tick densities as those observed in Ticino.

Borrelia infection rate using IF was 16% in *I. ricinus* adults and 10% in nymphs. These infection rates are lower than those previously reported by Miserez et al. [7] (25% in adults, 36.2% in nymphs) using IF but in this study ticks were collected both from vegetation and attached to hosts. Other infection rates using PCR (2%) and Real Time PCR (31, 65%) were reported in ticks collected in Ticino by Bernasconi et al. [8] and by Wicki et al. [14], respectively. A variation among results might be explained in part by differences in methodology used to determine the infection rate in ticks. In three localities, Bruzella, Morbio Superiore and Cugnasco, *I. ricinus* ticks were found free of *Borrelia* when tested by three different methods (IF, isolation and DNA detection by PCR of culture medium). Some other studies have also revealed a heterogeneous geographical distribution of *I. ricinus* and of the *Borrelia* infection prevalence in Switzerland [1].

Aeschlimann et al. [1] have demonstrated that the prevalence of *Borrelia* in ticks was correlated with altitude, but in our study this phenomenon is not observed. This might be due to the fact that the studied sites were geographically dispersed, this was not the case in the previous study [1], where ticks were collected at various altitudes on the slope of a single mountain.

IF was found to be more efficient than culture for detection of *Borrelia* in ticks. Similar observations were reported by Gern et al. [10]. Different hypotheses might explain this. The higher infection rate by IF than isolation might be related to heterogeneous distribution of the spirochetes in the ticks [15] and the fact that we used only one halved tick for isolation procedure could influence spirochete growth in BSK medium. In addition, the low number of spirochetes harbored in ticks as observed by IF can also explain this phenomenon. In fact, 96% of *I. ricinus* ticks examined were harboring < 50 spirochetes, only two ticks (4%) were infected by more than 500 spirochetes. This result can partly be responsible for the low isolation rate (2.4%) observed in *I. ricinus* ticks in canton Ticino. Comparison with results of another study performed in our laboratory using the same techniques [10] where the isolation rate reached 33%, allows to understand this low isolation rate. In fact, in this study, 36% of ticks were infected by a high density of spirochetes (more than 50 spirochetes) compared to 4% in the present study. Moreover, among the three *Borrelia* species observed in Ticino, two (*B. lusitaniae* and *B. valaisiana*) of them are recognized as difficult to isolate from ticks.

The use of IF and *Borrelia* isolation for each tick allows to count *Borrelia* in ticks, to isolate the bacteria, to characterize the *Borrelia* species and to detect and characterize *Borrelia* DNA in culture medium when no isolate has been obtained.

In the present study we observed that three genospecies of *B. burgdorferi* sl are present in canton Ticino: *B. garinii*, *B. valaisiana* and *B. lusitaniae*. In contrast, in a previous study in this part of Switzerland only DNA of *B. valaisiana* (VS116) had been detected by PCR in ticks collected from hosts [8]. Our data show that *B. garinii* and *B. lusitaniae* are also infecting ticks in canton Ticino, and *Borrelia* isolates from the three species could be obtained from ticks in the present study. Apparently *B. afzelii* and *B. burgdorferi* s.s. are absent from the studied sites. Additional studies are needed to confirm this especially because a study in canton Valais (in alpine valleys) by Péter et al. [6] showed the presence of *B. afzelii*, *B. burgdorferi* s.s., *B. garinii* and *B. valaisiana*.

B. garinii is the most frequent species observed in the studied areas, the same result was reported in Belgium [16], in Germany [17] and in various areas in Europe [18]. In contrast, in Slovakia [10] and in Finland [19], *B. afzelii* was the predominant genospecies, whereas in Ireland [20], in Portugal [21] and in Tunisia [22] *B. valaisiana* and *B. lusitaniae*, respectively were the most prevalent species. The heterogeneous distribution of *B. burgdorferi* sl could be associated with the diversity of reservoir hosts. A mixed infection with *B. garinii* and *B. valaisiana* has been found in one nymphal tick. De Michelis et al. [21] have reported that *B. garinii* and *B. valaisaina* constituted the majority of multiple infections. Additionally these two species have been described to infect the same reservoir host and to present a similar distribution [17, 23, 24]. *B. lusitaniae* has been previously reported in Portugal [25], in the Czech Republic, Moldavia, Ukraine [26], Slovakia [10], Tunisia [27] and Poland [28]. In our study, this species is described and isolated for the first time in Switzerland.

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