INCIDENCE OF *BORRELIA BURGDORFERI* S.L. AND ITS GENOMOSPECIES IN TICKS (*IXODES RICINUS*) COLLECTED IN PRAGUE 1994-1997

JIŘÍ BAŠTA, JIŘÍ PLCH, AND DAGMAR HULÍNSKÁ
National Institute of Public Health, Prague, Czech Republic

Abstract - A total of 12,911 ticks were collected by flagging from 10 localities of the city Prague between 1994-1997. The detection of *Borrelia burgdorferi* s.l. was performed using the indirect immuno-fluorescence method (with polyclonal hyperimmune serum) and 635 ticks collected in a urban park were selected for polymerase chain reaction detection. The tick infestation observed by indirect immuno-fluorescence ranged from 3.9% (1996) to 8.9% (1995), while polymerase chain reaction revealed from 3.4% (1996) to 9.2% (1995). *Borrelia garinii* moderately dominated, *Borrelia afzelii* was the second genomospecies detected. *Borrelia burgdorferi* sensu stricto has not been detected yet. Mixed infection *B. garinii*/ *B. afzelii* was recorded in one adult tick in 1997. Significant differences in tick infestation were observed between the seasons. No significant differences were found between the localities studied and their types (park, wooded park, wood) within one season. The results are indicative of the risk of acquiring Lyme borreliosis directly in the city.
Key words - Lyme borreliosis, epidemiology, immunofluorescence

Spirochetes *Borrelia burgdorferi* s.l. were identified as the causative agent of a multisystemic disease called Lyme borreliosis (LB) (Burgdorfer, 1984). The main vectors of this pathogen are ticks of the genus *Ixodes*, *Ixodes ricinus* (L.) is the species implicated in LB in the Czech Republic (CZ). Lyme borreliosis is the most frequent tick-borne infection notifiable in this country. The second one is tick-borne encephalitis. As many as 4,063 cases of LB were reported in 1994, compared to 619 cases of tick-borne encephalitis. An uprising trend was observed from 1989 to 1995 when 6,320 and 744 cases of LB and tick-borne encephalitis were reported, respectively. The LB incidence then decreased to 4,193 in 1996 and to 2,450 in 1997. The number of reported cases of tick-borne encephalitis showed the same tendency, 571 cases in 1996 and 415 in 1997 (EPIDAT, NIPH, Prague) (Hulínská *et al.*, 1995).

Findings of ticks and borreliae in the urban environment were reported (Kahl *et al.*, 1989). The mean infestation of ticks with *Borrelia* sp. widely varies, between 1.9%, 10.8% and 17.4% for larvae, nymphs and adults, respectively (Hubálek and Halouzka, 1998a). *B. burgdorferi* s.l. was divided into genomospecies using the genome analysis (*B. burgdorferi* s.s., *B. garinii*, *B. afzelii*, *B. lusitaniae* and *B. valaisiana*, are the genomospecies found in Europe) (Baranton *et al.*,1992; Le Fleche *et al.*, 1997; Wang *et al.*, 1997). The polymerase chain reaction (PCR) is the sensitive and specific method (Schmidt, 1997), which allowed differentiation between the genomospecies in the vector (Rijpkema *et al.*, 1995).

The aims of the study were to establish the incidence of *B. burgdorferi* s.l. and its genomospecies in ticks collected in urban environment in the different years and to compare the seasonal incidence in the same localities.

MATERIAL AND METHODS

Ticks were collected by flagging from shrubwood and open grass areas during the vegetation period, in the following localities: urban parks, Petřín, Vítkov, Stromovka; park-like woods (wooded park) Krč, Šárka, Vítkov, Hanspaulka, Hvězda, Hostivař and wood Ďáblice, Klánovice, Troja. These localities were described previously (Pokorný, 1990; Plch and Bašta, 1999).

Between 1994-1997, a total of 12,911 ticks were examined. The detection of *B. burgdorferi* s.l. was performed using the indirect immuno-fluorescence method (NIF) (Plch and Bašta, 1999) and 635 ticks collected in the locality Stromovka were selected for polymerase chain reaction (PCR) detection.

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These ticks were stored in a freezer at -19 ºC to be removed individually to sterile microcentrifuge tubes (Eppendorf) and homogenised by sterile glass pestles. Isolation of DNA was performed using the glass fiber fleece system, commercially available (Boehringer Manheim), according to the manufacturer´s instructions. The DNA yield and purity were measured using the spectrophotometer Ultrospec 2,000 (Pharmacia). DNA with the index of A260/280 nm 1.7-1.9 was used for the reactions. The primers detecting *B. burgdorferi* s.l. (LD) and differentiating its genomospecies (BB-*B. burgdorferi* s.s., BG-*B. garinii*, VS-*B. afzelii*) synthesised by I.D.T. (USA) were used in the single-step PCR as described previously (Marconi and Garon, 1992). The number of cycles was extended to 45 to increase the yield of the reactions. The reaction mixture in the 50 ml volume contains: PCR buffer (Gene-Amp, Perkin-Elmer) supplemented with 2.5 m M MgCl2, 200 m M of each d NTPs (d NTPs mix, Perkin-Elmer), 1,25 U Taq DNA polymerase (AmpliTaq, Perkin-Elmer) and 1-10 ml template DNA. Amplifications were carried out in the MJ Research PTC 200 Cycler (Biotech). Negative controls (reaction mixture lacking template) were included in each PCR, while 100 pg of aliquots DNA isolated from strains M192, Kc90, B31 (of different origins, NIPH, Prague; Hulínská *et al.*, 1993) were used as positive controls for each PCR. Sensitivity of the reaction was tested with different amounts of control DNA (5-520 fg), without and with tick DNA. Inhibition controls were performed the same way as positive controls, supplemented with template tick DNA.

The ticks selected for indirect immuno-fluorescence detection of spirochetes (12,276 ticks) were dipped into 70% alcohol and then washed with sterile PBS, the dorsum was removed and the midgut portion was extracted, transferred onto a slide and smeared. Air-dried specimens were fixed with acetone. Hyperimmune rabit serum prepared in our laboratory (immunisation with B31 strain, obtained by courtesy of Dr. A.G. Barbour, USA and Kc90 strain, National Reference Laboratory for Lyme borreliosis, NIPH, CZ) and FITC-labelled swine anti-rabbit immunoglobulin (SEVAC) were used for the detection of the spirochetes. The specimens were investigated under the fluorescence microscope (Olympus) at magnifications of 200 to 400x.

Statistical analysis

The study is based on the quantitative description of a population group, the percentage of frequency of each feature (P) was calculated together with its 95 % confidence interval (95 % CI, i.e. its upper and lower limits - UP, LP). Poisson distribution of the actual number of positive findings (x) was calculated for all the groups assessed (N). Diagrammatic representation of the confidence intervals enables comparison between the data. If these intervals do not overlap, the difference is statistically significant (minimally at a 5 % significance level) while overlapping intervals are indicative of a statistically insignificant difference between the frequencies compared, at a 95% confidence interval (Janko, 1958).

RESULTS

The infestation of ticks with *Borreliae* was expressed in percentages (Table 1). Collections of up to 50 ticks from one locality were not considered to be sufficiently significant. The *Borrelia* positivity rates in ticks ranged between 3.9% (1996) and 8.9% (1995). The overall incidence of ticks harbouring *Borrelia* was similar in all localities in the four seasons studied. The absolute number of the ticks infected was, as a rule, lower than 50, nevertheless, the percentages of the infected ticks in the selected localities were similar within the same season. This is suggestive of a high reliability of the estimate of the infection incidence and of a homogeneous distribution of the infection in all localities studied.

Detection limit of LD primer was found to be 52 fg of control borrelial DNA, in the presence of tick DNA 520 fg. The primers differentiating the genomospecies showed sensitivity of 35 fg (BG) and 350 fg (BB, VS) (5 fg represent aproximatly 1 spirochetal organism; Rijpkema *et al.*, 1995). The incidence rates of *B. burgdorferi* s.l. obtained by PCR were lower than these obtained by indirect immuno-fluo-

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rescence, with the exception of the adult ticks in 1995. No sample positive with the LD primer and negative with the others was found. The incidence rates obtained by PCR varied from 3.4 to 9.2%. Statistical analysis showed significant differences in the incidence, c2 =8.74 (P = 0.033). Adult ticks were significantly more frequently infected than nymphs (Table 2).

Numbers of positive findings of *Borrelia garinii* and *Borrelia afzelii* were: 7 compared to 5 in 1995, 6 compared to 5 in 1996 and 5 compared to 3 in 1997, respectively. The genospecies *Borrelia burgdorferi* s.s. was not detected in all years during the study. Mixed infection of *B. garinii / B. afzelii* was found in one adult ticks in 1997.

Table 1. Incidence of *Borrelia* sp. in *Ixodes ricinus* ticks collected in the city of Prague, Czech Republic between 1994-1997, detected by indirect immunofluorescence.

Year	Number of ticks				Positive findings %				95% C.I. (% of total)	
	nymphs	females	males	total	nymphs	females	males	total	LP	UL
1994	870	1,207	901	2,978	5.5	6.5	6.2	6.0	5.1	6.8
1995	1,312	1,103	737	3,152	8.0	12.2	9.5	9.9	8.9	10.5
1996	751	730	993	2,474	3.0	5.0	5.8	4.7	3.9	5.5
1997	976	1,498	1,212	3,686	7.0	5.6	6.9	6.5	6.2	7.8

C.I.= 95% confidence interval
LP = lower limit of C.I.
UP =upper limit of C.I.

Table 2. Incidence of *Borrelia burgdorferi* sensu lato in *Ixodes ricinus* ticks collected in the urban environment between 1995-1997, detected by PCR (LD primer).

Year	Number of ticks			Positive findings %			95% C.I. (% of total)	
	nymphs	adults	total	nymphs	adults	total	LP	UL
1995	103	27	130	2.9	33.3	9.2	4.9	15.6
1996	235	91	326	1.7	7.7	3.4	1.5	6.5
1997	124	55	179	1.6	10.9	4.5	1.9	8.6

C.I.= 95% confidence interval
LP = lower limit of C.I.
UP =upper limit of C.I.

DISCUSSION

The infestation of ticks, collected in the same localities of the same city with the causative agent of LB was also observed using indirect immuno-fluorescence (Plch and Bašta, 1999). The results are consistent with these of the previous study, nevertheless, the use of the but the PCR for detection of *B. burgdorferi* s.l. proved a higher specificity and allowed genomospecies identification. In all localities, the ticks were infected with a roughly equal probability (data not shown). Significant differences

were found between different seasons. Any significant differences in the infection incidence in ticks depending on the type of the wooded area have not been demonstrated.

The tendency of PCR detection to miss some of *Borrelia* infection in ticks was reported (Kahl *et al.*, 1998), which is consistent with the findings of the presented study. This observation could be explained by the presence of some inhibitors in ticks, which resulted in lower sensitivity of the reaction (Bašta and Hulínská, 1999). Unfortunately, the primers selected for the study in 1995 were unable to differentiate two newly described genospecies of *B. garinii* (LeFleche *et al.*, 1997; Wang *et al.*, 1997). Therefore the ratio *B. garinii* / *B. afzelii* could vary if different primers sets had been used.

The study is the first to record the distribution of different genospecies of *Borrelia burgdorferi* s.l. in the urban environment. A moderate domination of *Borrelia garinii* was revealed, consistent with other findings in Central European countries (Hubálek and Halouzka, 1998b). Any significant seasonal differences in the frequency of individual genospecies were not found. *Borrelia burgdorferi* sensu stricto has not been either detected or isolated in the Czech Rep. (Hulínská *et al.*, 1993; Hubálek and Halouzka, 1998b). This study support the hypothesis that this genospecies is not common in the CZ, which is not true for some localities in southern Europe (Cinco *et al.*, 1996a). In Austria, *Borrelia burgdorferi* s.s. is rather infrequent (Hubálek and Halouzka, 1998b). Mixed infection with two genomospecies was reported in one adult tick. Interestingly, much higher incidence of mixed infection was found in Italy near Trieste (Cinco *et al.*, 1996b), where tick infestation rates were higher in general.

The incidence of *Borrelia* infection in ticks should be always established in the same season. Since varies widely during the years. The results demonstrated the risk of acquiring Lyme borreliosis directly in the city of Prague. This risk is increased by persisting wrong habits in tick removal (Bašta *et al.*, 1998).

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