ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 2007, 14, 75-79

ISOLATION, CULTIVATION AND IDENTIFICATION OF *BORRELIA BURGDORFERI* GENOSPECIES FROM *IXODES RICINUS* TICKS FROM THE CITY OF BRNO, CZECH REPUBLIC

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Pejchalová K, Žákovská A, Mejzlíková M, Halouzka J, Dendis M: Isolation, cultivation and identification of *Borrelia burgdorferi* genospecies from *Ixodes ricinus* ticks from the city of Brno, Czech Republic. *Ann Agric Environ Med* 2007, **14**, 75-79.

Abstract: A total of 305 ticks (21 larvae, 243 nymphs, 19 females and 22 males) were collected by flagging of vegetation in suburban woods of Pisárky Park (city of Brno) from July to October 2002. The midgut of each tick was dissected out and transferred individually into BSK-H medium. After cultivation, all specimens were examined by dark-field microscopy (DFM) for the presence of borreliae. Out of 305 tick samples, 45 were (14.8%) DFM positive. The following polymerase chain reaction (PCR) then revealed 37 (12.1%) samples positive for the presence of *Borrelia burgdorferi* sensu lato DNA. All 37 samples were further analysed by restriction fragment length polymorphism (RFLP) method. PCR-RFLP analysis revealed 14 strains of *B. afzelii* (37.8%), 15 strains of *B. garinii* (40.5%) and 2 strains of *B. burgdorferi* sensu stricto (5.4%). Four samples (10.8%) showed a mixed population of these genospecies. Two samples produced atypical RFLP pattern which were detected by sequence analysis as *B. valaisiana* (5.4%). Isolation attempts resulted in 21 spirochaetal strains (including two stains of *B. valaisiana*). The results show the diversity of *B. burgdorferi* s.l. in tick population and refer the risk of infection by pathogenic borreliae in Brno.

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Key words: *Borrelia burgdorferi*, cultivation, DFM method, *Ixodes ricinus*, Lyme borreliosis, PCR method.

INTRODUCTION

Lyme borreliosis (LB) is a multisystemic infection caused by spirochetes of the genus *Borrelia*, acquired as a consequence of a bite from an infected tick [3]. Since its discovery in 1982, many strains of *Borrelia burgdorferi* have been isolated from ticks, vertebrate reservoir hosts and humans.

The genospecies responsible for human LB are *Borrelia burgdorferi* sensu stricto (s.s.), *Borrelia garinii* and *Borrelia afzelii* [22]. In Europe, *B. garinii* and *B. afzelii* are the most prevalent genospecies, whereas *Borrelia burgdorferi* s.s. is the only genospecies encountered in North America. Lyme borreliosis is the most common tick-borne disease in Eurasia [16] and North America [19].

The main vector of this infectious agent in the Czech Republic is *Ixodes ricinus* (L.), the most frequent tick in Europe. *Ixodes persulcatus* Schultze in Euroasia, Ixodes pacificus Cooley and Kohls and *Ixodes scapularis* Say in North America [1] are other proven vectors.

Received: 24 March 2006
Accepted: 25 August 2006

The ticks, including the infected ones, also occur regularly in urban forest parks in the neighbourhood of large cities [2, 21]. Generally, the presence of *B. burgdorferi* sensu lato (s.l.) in *I. ricinus* ticks has been reported from former Czechoslovakia [7, 8, 14] at a frequency up to 43.0% of ticks in particular localities. In subsequent years, surveys in the Czech Republic reported positivity of 30.7% in females, 27.4% in males and 17.1% in nymphs in South Moravia [12].

In the present study, we focused our attention on the distribution of *B. burgdorferi* s.l. in the Pisárky park locality situated in Brno city (South Moravia, Czech Republic) and on the occurence of *Borrelia*n genospecies in the tick population.

MATERIAL AND METHODS

Tick collection. Ticks were collected by flagging with flannel cloth over vegetation on selected area of 150 × 300 m from July – October 2002. Average temperature was 19ºC and average air humidity was 67%.

Locality. The Pisárky locality (49°11'30"N, 16°34'31"E) is a suburban park (district of Brno city) about 2 km from the centre of the city. It is situated 197-210 m above sea level and forms the bottom of Pisárky valley. A mixed wood is on the hillside of this valley and is used regularly for recreation. The predominant plants in this biotope have been described previously [23]. Local hosts of *I. ricinus* ticks at the locality include medium-sized and small mammals, such as hare *Lepus europaeus*, squirrel *Sciurus vulgaris*, hedgehog *Erinaceus concolor*, bank vole *Clethrionomys glareolus*, yellow-necked mouse *Apodemus flavicollis* and wood mouse *A. sylvaticus*.

Cultivation and microscopy. Collected ticks were placed into tubes and stored under cool (5°C) and humid (r.h. 90%) conditions until examination. For the detection of spirochetes, ticks were dissected, their midgut content was triturated individually on a sterile slide in a drop of BSK-H complete medium (Sigma, USA) and each specimen was transferred into its own BSK-H medium, supplemented with 6% of rabbit serum, rifampicin (50 µg/ml) and phosphomycin (100 µg/ml) to prevent bacterial contamination. Samples were cultivated at 33°C and after 10-14 days examined for the presence of spirochetes by dark-field microscopy (DFM) at 400× magnification. In the case of positive results the cultures were further passaged and 150 µl of medium from each tube was taken for identification by PCR-RFLP detection.

DNA purification. DNA of detected samples was isolated from homogenates using a DNA isolation kit (Malamité v.o.s., Czech Republic). This procedure is based on cell lyses by sarkosyl and chaotropic ions and subsequent binding of DNA in silica particles. DNA was eluted from silica particles in 20 µl of TE buffer (10 mM Tris-Cl, 1mM EDTA). 5 µl of this preparation was used for amplification.

PCR assay. Based on the specific flagellin sequence amplification for detection of *B. burgdorferi* s.l. was performed. The 50 µl PCR mixture contained: 1x HotStarTaq Master Mix (Qiagen, Germany), 15 pmol of each FL3 primer (5'-MGA GCT TCT GAT GAT GCT GCT GGY ATG GGR G-3') and FL5 primer (5'-GRG GAA CTT GAT TAG CYT GYG CAA TCA TTG CC-3'), 100 µM of dUTP (Sigma), and 5 µl of template DNA received after standard DNA isolation. All PCR runs were performed on a thermocycler (PTC-200, MJ Research) with the following profile: an initial activation step at 96°C for 12 min, 30 cycles consisting of a denaturation step for 10 sec at 96°C, an annealing step for 10 sec at 65°C, an extension step for 40 sec at 72°C and the final extension at 72°C for 4 min.

RFLP. Restriction analysis of amplified PCR products was performed by Alu1 endonuclease digestion (New England BioLabs, USA). The restriction fragments were separated on a 2% (w/v) agarose gel, stained by ethidium bromide staining, visualised by UV transillumination (312 nm), and analysed by ULTRA LUM (Ultra-Lum, Inc., USA) gel detection and analysis system. The typical restriction patterns characterictic for *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* were identified [13].

Sequencing. The amplifications products of 2 samples with atypical RFLP patterns were sequenced from both sides by using FL3 and FL5 primers, respectively. Achieved sequences were processed by "Sequencing-Service" of Faculty of Science, Masaryk University (Brno, Czech Republic). Using the Blast algorithm, a search for the best sequence-homologies was performed.

RESULTS

A total of 305 *I. ricinus* ticks (21 larvae, 243 nymphs, 19 females and 22 males) were collected and examined by DFM method (Tab. 1) in the period of late summer and early autumn (July – October) 2002. Motile spirochetes, morphologically resembling *Borrelia*e were observed in 14.8% (45 ex.) of all samples: 9.5% (2 ex.) larvae, 14.4% (35 ex.) nymphs, 26.3% (5 ex.) females and 13.6% (3 ex.) males. Out of 45 DFM positive specimens, 37 (12.1%) samples were PCR positive for the presence of *B. burgdorferi* s.l. DNA. All these samples were further analysed

Table 1. Prevalence of *Borrelia*e in tick stages examined by dark-field microscopy (DFM) and polymerase chain reaction (PCR) including isolated strains.

Stage	*I. ricinus* n (%)	DFM positive n (%)	PCR positive n (%)	Isolated strains n (%)
Larvae	21 (6.9)	2 (4.4)	0 (0.0)	0 (0.0)
Nymphs	243 (79.7)	35 (77.8)	34 (91.9)	19 (90.5)
Females	19 (6.2)	5 (11.1)	2 (5.4)	1 (4.8)
Males	22 (7.2)	3 (6.7)	1 (2.7)	1 (4.8)
Total	305	45 (14.8)	37 (12.1)	21 (6.9)

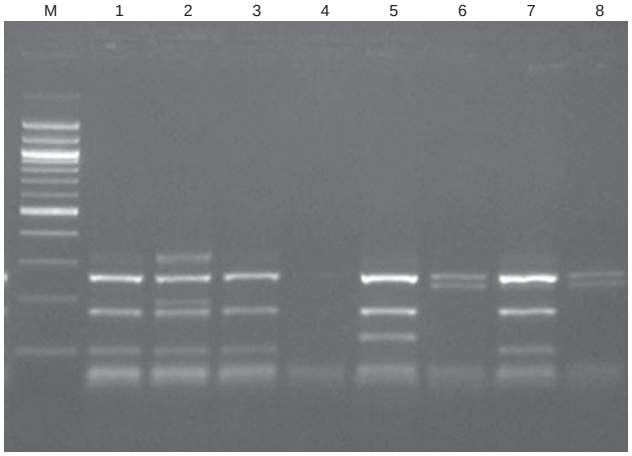

Figure 1. Resulting RFLP patterns obtained after *Alu*1 endonuclease treatment of amplified specific *B. burgdorferi* s.l. flagellin sequence products. Agarose gel electrophoresis. 1 – BRZ23 (*B. garinii*), 2 – BRZ36 (mixture of *B. burgdorferi* s.s. and *B. garinii*), 3 – BRZ25 (*B. garinii*), 4 – negative sample, 5 – BRZ34 (*B. valaisiana*), 6 – BRZ30 (*B. afzelii*), 7 – BRZ32 (*B. garinii*), 8 – BRZ33 (*B. afzelii*), M – 100 bp molecular weight standard (Malamité).

by PCR-RFPL methods. RFLP analysis revealed 14 strains of *B. afzelii* (37.8%), 15 strains of *B. garinii* (40.5%) and 2 strains of *Borrelia burgdorferi* sensu (5.4%) stricto. Four samples (10.8%) showed a mixed culture: 2 specimens *B. afzelii* and *B. garinii*, 1 specimen *B. afzelii* and *B. burgdorferi* s.s. and 1 specimen *B. garinii* and *B. burgdorferi* s.s. Two samples (X58 and X94) produced atypical RFLP pattern (Fig. 1). Direct sequencing of amplified product of flagellin gene of these samples revealed the highest (96%) homology with B. valaisiana (5.4%). From all DFM positive samples (45) we obtained 21 spirochetal strains and they were all identified as *B. burgdorferi* s.l. with a 46.7% success in cultivation (Tab. 2).

DISCUSSION

The aim of our study was to determine the occurrence of *B. burgdorferi* s.l. and its genospecies in *I. ricinus* ticks in the urban park of Pisárky, used frequently for recreation by inhabitants of Brno, the second largest city in the Czech Republic. The appearance of ticks in cities seems to be connected with the establishing of new parks in original forest areas, and with building new residential districts on the peripheries of towns close to forests. The persistence of ticks at the given locality is determined by the possibility to realize their entire life cycle which, after all depends, upon the presence of suitable hosts for all developmental stages. The occurrence of ticks has a mosaic-like character and their infection with *Borrelia*e at different places of certain area with the presence of LB agent is highly variable, depending on locality and time of investigation [17].

A number of investigations have been performed in Pisárky for several years. During the period 1996-2002, a number of surveys were conducted at regular intervals and revealed a different prevalence of *B. burgdorferi* s.l.

Table 2. Summary results of all DFM positive samples, PCR-RFLP analysis and attempts of isolation.

Samples	Stage	PCR-RFLP	Marked isolated strains
X4	N	*B. afzelii* + *B.b.s.s.*	–
X5	N	*B. garinii*	BRZ23
X8	N	*B.b.s.s.*	BRZ24
X12	N	*B. garinii*	BRZ25
X16	N	*B. garinii*	BRZ43
X18	F	*B. garinii*	BRZ26
X19	N	*B. garinii*	BRZ27
X22	N	*B. afzelii*	–
X23	M	*B. garinii*	BRZ28
X24	N	*B. garinii* + *B. afzelii*	BRZ29
X27	N	*B. afzelii*	BRZ30
X28	N	*B. afzelii*	BRZ31
X33	N	*B. afzelii*	–
X41	N	*B. garinii*	BRZ32
X54	N	*B. afzelii*	BRZ33
X58	N	*B. valaisiana*	BRZ34
X59	N	*B. afzelii*	–
X94	N	*B. valaisiana*	BRZ35
X125	L	Neg.	–
X129	F	Neg.	–
X144	F	Neg.	–
X64	N	*B. garinii*	–
X65	N	*B. garinii*	–
X67	M	Neg.	–
X72	F	*B. garinii*	–
X76	M	Neg.	–
X80	L	Neg.	–
X164	F	Neg.	–
X166	N	*B. garinii* + *B.b.s.s.*	BRZ36
X183	N	Neg.	–
X192	N	*B. garinii*	BRZ37
K3	N	*B. afzelii*	–
K5	N	*B. afzelii*	–
K8	N	*B. afzelii*	–
K10	N	*B. afzelii*	–
K16	N	*B. garinii*	BRZ38
K46	N	*B. garinii*	BRZ39
K63	N	*B. afzelii*	–
K69	N	*B. afzelii*	–
K79	N	*B. garinii*	BRZ40
K81	N	*B. afzelii* + *B. garinii*	BRZ41
K85	N	*B. afzelii*	–
K90	N	*B.b.s.s.*	–
K94	N	*B. garinii*	BRZ42
K96	N	*B. afzelii*	–
Total		37	21

L – larva, N – nymph, F – female, M – male

(mean positivity 6.5%) from March – November. The findings showed a cyclical character of positivity. From 1996 when 6.3% of positive ticks were detected, the positivity rose to 9.7% in 1997 and 12.3% in 1998. Then the number went down steeply to 3.6% in 1999 and subsequently increased to 4.0% in 2000 [13], 5.5% in 2001 and 6.4% in 2002 (March – June; Mejzlíková, not published).

In our precedent studies, samples were detected by using DFM at first and positive samples were futher investigated by PCR methods without previous cultivation. Since we cultivated the samples prior to examination by DFM, the mean infection rate was significantly higher and reached 14.8% (DFM) or 12.1% (PCR). The prevalence in females (26.3%) was found to be statistically higher than the prevalence in nymphs (14.4%), which is in agreement with the general pattern of increasing *Borrelia* prevalence through the life stages of ticks [11]. The success of isolation attempts reached 46.7%. We presume that this high success rate was due to the direct transferring of the gut contents into the culture medium.

In an analogical study made in Brno city parks, Hubálek [9] observed an average prevalence of 27.0% (29.7% females, 30.8% males and 14.7% nymphs). Other studies from urban environment have been in Prague (Czech Republic) and Košice (Slovakia). Variability of infection was from 3–16% during the years 1996 and 1998 [2, 21].

Our findings and the results of the other authors confirm the considerations on the geographical variability of *B. burgdorferi* s.l. strains isolated in Europe. *B. afzelii* and *B. garinii* commonly occure in different European countries [6, 10, 15], including the Czech Republic [2, 13]. *B. valaisiana* has already been reported from the Czech Republic [5], but has been isolated for the first time.

This study supports the hypothesis that *B. burgdorferi* s.s. is quite rare in the Czech Republic. The only finding of *B. burgdorferi* s.s. from Czech Republic was reported from South Bohemia [22]. In Austria, *B. burgdorferi* s.s. was detected also infrequently [10]. However, opposite results were shown in some localities in southern Europe [4]. Our examination revealed two findings of *B. burgdorferi* s.s. and two mixed infections of *B. burgdorferi* s.s. with *B. garinii* and *B. afzelii*. Another two mixed infections within two genospecies, *B. afzelii* and *B. garinii*, were disclosed, thus a double infection occured in 11.4% of ticks. A lower incidence (5.7% of positive ticks) of double infection has also been detected in the urban environment of Prague (Czech Republic) [2]. A triple infection of *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* is documented in Slovakia [18].

CONCLUSIONS

The observation presented in this report contributes to the knowledge about the prevalence of *B. burgdorferi* genospecies in *I. ricinus* ticks in Brno city, Czech Republic. The results indicate high probability of human contact with the causative agent of Lyme disease in the urban park of Pisárky and thus the considerable risk of infection. The important part of the study is the first report of *B. valaisiana* detection in South Moravia and its first successful laboratory cultivation in the Czech Republic.

Acknowledgements

This work was supported by MSM: 0021622415 and partially by FRVŠ 2548/G3.

REFERENCES

1. Anderson J: Epizootiology of *Borrelia burgdorferi* in *Ixodes* tick vector and reservoir hosts. Rev Infect Dis 1989, 11, 1451-1459.
2. Bašta J, Plch J, Hulínská D, Daniel M: Incidence of *Borrelia garinii* and *Borrelia afzelii* in *Ixodes ricinus* ticks in an urban environment, Prague, Czech Republic, between 1995 and 1998. *Eur J Clin Microbiol Infect Dis* 1999, **18**, 515-517.
3. Burgdorfer W, Barbour AG, Hayes SF, Benach JL, Grunwaldt E, Davis JP: Lyme disease – a tick-borne spirochetosis? *Science* 1982, **216**, 1317-1319.
4. Cinco M, Padovan D, Murgia R: Detection by PCR of different species of *Borrelia burgdorferi* in *Ixodes ricinus* collected in defined area of northern Italy. *Parassitologia* 1996, **38**, 378.
5. Derdáková M, Beati L, Peťko B, Stanko M, Fish D: Genetic variability within *Borrelia burgdorferi* sensu lato genospecies established by PCR-single-strand conformation polymorfism analysis of the *rrfA-rrlB* intergenic spacer in *Ixodes ricinus* tick from the Czech Republic. *Appl Environ Microbiol* 2003, **69**, 509-516.
6. Gern L, Hu CM, Kociánová E, Výrosteková V, Reháček J: Genetic diversity of *Borrelia burgdorferi* sensu lato isolates obtained from *Ixodes ricinus* ticks collected in Slovakia. *Eur J Epidemiol* 1999, **15**, 665-669.
7. Hubálek Z, Korenberg EI, Juřicová Z, Kovalevskij JV, Halouzka J, Ščerbakov SV: Prevalence of *Borrelia*e in *Ixodes ricinus* ticks from southern Moravia, Czechoslovakia. *Folia Parasitol* 1990, **37**, 359-362.
8. Hubálek Z, Halouzka J, Juřicová Z: A comparison of the occurrence of borreliae in nymphal and adult *Ixodes ricinus* ticks. *Zentralbl Bakteriol* 1991, 275(1), 133-137.
9. Hubálek Z, Halouzka J, Juřicová Z: Prevalence of *Borrelia*e in *Ixodes ricinus* ticks from urban parks. *Folia Parasitol* 1993, **40**, 236.
10. Hubálek Z, Halouzka J: Distribution of *Borrelia burgdorferi* sensu lato genomic groups in Europe, a review. *Eur J Epidemiol* 1997, **13**, 951-957.
11. Hubálek Z, Halouzka J: Prevalence rates of *Borrelia burgdorferi* sensu lato in host-seeking *Ixodes ricinus* ticks in Europe. *Parasitol Res* 1998, **84**, 167-172.
12. Hubálek Z, Halouzka J, Juřicová Z: Longitudinal surveillance of the tick *Ixodes ricinus* for *Borrelia*e. *Med Vet Entomol* 2003, **17**, 46-51.
13. Janouškovcová E, Žákovská A. Halouzka J, Dendis M: Occurence of *Borrelia afzelii* and *Borrelia garinii* in *Ixodes ricinus* ticks from southern Moravia, Czech Republic. *Vector Borne Zoonotic Dis* 2004, **4**, 43-52.
14. Kmety E, Řeháček J, Výrosteková V: Investigation of ticks for the presence of *Borrelia* in Czechoslovakia. *Zentralbl Bakteriol* 1986, **263**, 468-470.
15. Kurtenbach K, De Michelis S, Sewell HS, Etti S, Schafer SM, Hails R, Collares-Pereira M, Santos-Reis M, Hanincová K, Labuda M, Bormane A, Donaghy M: Distinct combinations of *Borrelia burgdorferi* sensu lato genospecies found in individual questing ticks from Europe. *Appl Environ Microbiol* 2001, **67**, 4926-4929.
16. O`Connell S, Granstrom M, Gray JS, Stanek G: Epidemiology of European Lyme borreliosis. *Zentralbl Bakteriol* 1998, **287**, 229-240.
17. Peťko B, Siuda K, Stanko M, Tresová G, Karbowiak G, Fričová J: *Borrelia burgdorferi* sensu lato in the *Ixodes ricinus* ticks in Southern Poland. *Ann Agric Environ Med* 1997, **4**, 263-269.
18. Schwarzová K, Čižnár I: Combined infection of *Ixodes ricinus* with three *Borrelia burgdorferi* sensu lato genotypes. *Folia Microbiol* 2004, **49**, 297-300.

19. Steere AC: Lyme disease: a growing threat to urban populations. Proc Natl Acad Sci USA, 1994, 91, 2378-2383.

20. Štěpánová-Tresová G, Kopecký J, Kuthejlová M: Identification of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia afzelii* in *Ixodes ricinus* ticks from Southern Bohemia using monoclonal antibodies. *Zentralbl Bakteriol* 1999, **289**, 797-806.

21. Štěpánová-Tresová G, Peťko B, Štefančíková A, Nadzamová D: Occurence of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* and *Borrelia afzelii* in the *Ixodes ricinus* ticks from Eastern Slovakia. *Eur J Epidemiol* 2000, **16**, 105-109.

22. Wang G, Van Dam AP, Schwartz I, Dankert J: Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. *Clin Microbiol Rev* 1999, **12**, 633-653.

23. Žákovská A, Dendis M, Pejchalová K: Spirochaetes in Aedes species, *Culex pipiens pipiens* larvae and hibernating *Culex pipiens molestus* mosquitoes detected with dark-field microscopy (DMF) and polymerase chain reaction (PCR) methods. *Biológia* 2000, **55**, 667-670.