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Phenotypic and Genotypic Analysis of *Borrelia* spp. Isolated from *Ixodes ricinus* Ticks by Using Electrophoretic Chips and Real-Time Polymerase Chain Reaction

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ABSTRACT. The genotype of *Borrelia burgdorferi* sensu lato was detected in 371 out of 1244 ticks. *Borrelia* determination was based on partial sequencing of the 16S rRNA gene and real-time polymerase chain reactions for identification and quantitation of *ospA* and *recA* genes. Different *Borrelia* spp. were identified; *B. garinii* in 40 % ticks followed by *B. afzelii* (36.3 %), *B. burgdorferi* sensu stricto (12.9 %), *B. valaisiana* (3.5 %), *B. lusitaniae* (0.8 %), *B. bissettii* (0.5 %) and *B. miyamotoi*-like (0.5 %). Cultivation of 30 borrelia strains in BSK-H medium, among them *B. valaisiana*, *B. bissettii*-like and *B. miyamotoi*-like strains was unique in Czechia. Calibrated microfluidic-based quantification showed differences in the concentration of the nucleic acids and molar mass of the outer surface proteins of different *Borrelia* spp. with standard sensitivity and specificity and was helpful for their identification. The outer surface protein OspA was absent in *B. miyamotoi*-like and the OspB protein in *B. valaisiana*, *B. lusitaniae* and in three subtypes of *B. garinii*.

Abbreviations

Bbl	*Borrelia burgdorferi* sensu lato	*Bbs*	*B. burgdorferi* sensu stricto
BSK	Barbour–Stoenner–Kelly medium	OspA, OspB, OspC	outer surface proteins A, B, C
DFM	dark-field microscopy	PBS	phosphate-buffered saline
EE	Experion electrophoretic (chips)	qPCR	quantitative polymerase chain reaction
EM	erythema migrans	RT-PCR	real-time PCR
LB	Lyme borreliosis	SDS	sodium dodecylsulfate

Hard ticks (family *Ixodidae*) transmit many pathogens of the genera *Borrelia* and *Anaplasma* known to be causative agents of LB and human granulocytic anaplasmosis. LB, the most prevalent in Europe and North America, is caused by spirochetes of the *B. burgdorferi* sensu lato complex including at least 10 species. *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii* (Baranton *et al.* 1992), *B. valaisiana* (Wang *et al.* 1997) and *B. lusitaniae* (Hubálek *et al.* 1996; Le Fleche *et al.* 1997; Derdáková *et al.* 2003; Collares-Piera *et al.* 2004) are pathogenic for humans in Europe. Two additional *Borrelia* spp. were found: *B. bissettii* (Postic *et al.* 1998) isolated from Slovenian ticks (Picken *et al.* 1999) and *Borrelia* classified closer to the relapsing fever group *B. lonestari* (Amstrong *et al.* 1996; Rich *et al.* 2001), transmitted by hard ticks. Richter *et al.* (2003) have shown that relapsing fever-like borrelia infected 3.5 % of questing *Ixodes* ticks in central Europe. *B. miyamotoi*, transmitted by *I. persulcatus* in Asia (Fukunaga *et al.* 1995; Scoles *et al.* 2001) has been detected in Swedish *Ixodes ricinus* ticks (Fraenkel *et al.* 2002).

A sensitive and reliable method for genotyping strains of the genus *Borrelia* was established to be RT-PCR (Morrison *et al.* 1999; Pietila *et al.* 2000; Mommert *et al.* 2001; Hulínská *et al.* 2004; Rudenko *et al.* 2005) and multiplex RT-PCR (Courtney *et al.* 2004).

The aim of this study was to use RT-PCR analysis and to compare the results with new Experion electrophoretic (EE) chips for a rapid, sensitive and specific evaluation of the genetic and antigenic extent of heterogeneity within the *B. burgdorferi* sensu lato complex isolated from *I. ricinus* ticks. The application of calibrated quantification of partial fragments of *ospA* gene and comparison with the quantity of antigens could help in the development of a vaccine and of diagnostic procedures. This comparative technology has not been used for *Borrelia* sp. so far.

MATERIALS AND METHODS

Tick collection. Questing 1244 *I. ricinus* adult and nymphal ticks were collected during spring–summer seasons of 2003–2005 in 13 endemic localities of 8 Czech regions (Table I). Collections were predominantly performed by flagging. Eight nymhal ticks were removed from a woman returning from Magnitogorsk (Russia). Study sites were located mainly in the mixed deciduous forests where there previously the highest occurrence of *Borrelia* spp. in animals had been observed (Hulínská *et al.* 2002). Ticks from different localities were placed separately in 5-mL tubes containing grass. A total of 594 adults and 240 out of 650 nymphs were examined individually. The rest of the nymphs were examined in 90 pools of 3 ticks and 70 of 2 ticks. In the laboratory they were washed with 60 % ethanol and with PBS, then transferred to Eppendorf vials and partially homogenized by scissors and pestles with 0.5 mL of PBS; all steps were carried out under sterile conditions. A portion was screened by DFM; the others by cultivation and PCR.

Table I. PCR screening of DNA of *B. burgdorferi* sensu lato in *I. ricinus* ticks

No.	Locality	Number of			Positive adults %	Positive nymphs %	Total-to-positive tick number	Prevalence of positive ticks, %
		tick females to positive females	tick males to positive males	nymphs to positive				
1	Lysá-Dvorce	35/9	10/2	18/6	24.4	33.3	63/17	26.9
2	Mladá Boleslav	25/10	6/0	22/6	32.2	27.2	53/16	30.1
3	Hradec Králové	45/14	6/1	50/15	29.4	30.0	101/30	29.7
4	Pardubice	40/12	7/1	32/10	27.6	31.2	79/23	29.1
5	Karlovy Vary	45/16	5/0	74/18	35.5	24.3	124/34	27.4
6	Třeboň	26/8	3/1	60/13	31.0	21.6	89/22	33.8
7	Ždár nad Sázavou	46/11	0	40/16	23.9	40.0	86/27	31.3
8	Bor u Skuče	38/14	10/1	50/15	31.2	30.0	98/30	30.5
9	Nový Jičín	34/11	6/2	58/13	22.4	34.2	78/26	33.3
10	Frýdek-Místek	42/17	8/1	80/24	36.0	30.0	130/36	27.6
11, 12	Lučina, Ostrava	38/12	9/2	55/17	31.5	30.9	87/29	33.3
13	Brno-Sokolské	48/20	9/1	56/16	41.6	28.5	113/45	39.8
14	Napajedla	43/16	10/1	55/19	32.0	34.5	108/36	33.3
Total		505/170	89/13	650/188	30.8	28.9	1244/371	29.8

Cultivation of Borrelia *and preparation of antigens.* Cultures, control and derived *Borrelia* strains were maintained according to Barbour (1984), Hubálek *et al.* (1990) and Hulínská *et al.* (1992, 1998). Among the strains used 30 were isolated from ticks in the period of 2003–2005. Culture was performed from 321 DFM-positive samples in BSK-H medium (*Sigma*), with 7 % rabbit serum (*Sigma*) and 1.5 % Bacto gelatin (*Difco*, Germany). Rifampicin (*Sigma*) (30 μg/mL) was used. Cultures were incubated at 34 °C and growth was determined using DFM.

Protein analysis was performed from aliquots of strains refrigerated at –70 °C. They grew in 10 mL screw cap tubes Cellstar (*Greiner Bio-One International*) with BSK-H medium at 34 °C and then were transferred for 4 d to 200 mL of BSK-H medium. The borreliae were harvested by centrifuging (8000 ***g***, 30 min, 12 °C), washing twice in PBS-Mg^{2+} and redistilled water. The suspension obtained was sonicated on ice at 90 Hz for 5 min and then centrifuged (10 000 ***g***, 15 min, 4 °C). Each supernatant was designated as antigen of the recognized strain and stored at –70 ° C.

Preparation of DNA and genotyping. DNA was isolated using a QIAamp DNA Micro Kit (*Qiagen*), as recommended by the supplier, with prolonged incubation with proteinase K. Samples were first screened for borrelial DNA by PCR using primers targeting 16S rRNA and *ospA* genes (Richter *et al.* 2002; Fraenkel *et al.* 2002; Hulínská *et al.* 2006). *Borrelia* strains N40, HT31 kindly provided by B. Luft (*Stony Brook University*, USA) and our previously isolated strains 192M, 93M, K_c90, E5.seg, E51 and E50 from cerebrospinal fluid (M), blood (K_c) and erythema migrans (E) were used as positive controls. Master mixture for amplification in MJ PTC-200 thermocycler (*MJ Research*, USA) was described by Hulínská *et al.* (1999).

RT-PCR methods for *Borrelia* identification to the species level was performed by methods utilizing *ospA* sequences (Rauter *et al.* 2002; Godfroid *et al.* 2003) and a sequence of *recA* gene (Pietila *et al.* 2000) by LightCycler (*Roche Diagnostics*, Germany). Quantitative analysis of *Borrelia* was performed by qPCR with *recA* primers. We used *B. garinii* 192M strain with DNA concentration of 2.2 μg/mL (*i.e.* 2.2 ng per

μL) as a control for qPCR. Sensitivity was measured at DNA concentration ranging from 4 ng to 40 fg per μL. Light Cycler FastStart Master SYBR Green 1 kit (*Roche Diagnostics*) was used according to the instructions of the manufacturer. The reaction volume in a glass capillary contained 2 μL Master SYBR Green I mix (*Roche Diagnostics*), 2 μL of each primer (all 0.8 μmol/L), 2.5 μL of 25 mmol/L $MgCl_2$, 11 μL of PCR grade water and 2.5 μL of the template DNA at different dilutions. Melting curves were acquired for *ospA* and *recA* according to Rauter *et al.* (2002) and Pietila *et al.* (2000).

Partial sequencing of the *ospA* of *Borrelia* spp. and the 16S rRNA genes was used for the evaluation of genotypes in the phylogenetic trees (Hulínská *et al.* 2006). Sequences were aligned using the Clustal W program. Phylogenetic trees were generated with TREECON software. The following *GenBank* accession numbers were used to retrieve the corresponding 16S rRNA sequences: X98231, X98229 for *B. lusitaniae* (strain BR41), X98232 for *B. valaisiana* (strain VS116), X85195 for *B. garinii* (strain PBi), M64311 for *B. garinii*, X85191 for *B. afzelii* (strain DK21), X85195 for *B. burgdorferi* (strain DK27), L40596, AJ224138 for *B. bissettii* (strain DN127), D45192 for *B. miyamotoi*. Sequences of partial *ospA* genes of our *Borrelia* spp. were deposited in *GenBank* under accession numbers DQ005955, DQ064600, DQ064002, DQ064003, DQ650327, EF364112, EF364113, EF364114, EF364115.

Experion electrophoretic (EE) chip analysis. Proteomic expression analysis was made in 30 *Borrelia* strains isolated from ticks and from controls. EE chips integrated the separation, detection and data analysis of different antigens in accordance with their molar mass ranging from 10 to 260 kDa. We used Pro260 kit (Experion; *BioRad*, USA) as recommended by the supplier. The Pro260 ladder contains nine recombinant proteins. Two standards, lower and upper markers were used for the proper alignment of samples (*see* Table III). Preparing the gel stain and priming the filtered gel into the chip was done according to supplier's recommendations.

Samples (4 μL) were mixed with 2 μL sample buffer (Pro260) containing 2-sulfanylethanol ('2-mercaptoethanol') and SDS and boiled for 3–5 min, cooled, then spun down for 20 s, and 80 μL of deionized water was added to each tube and briefly vortexed. After priming, the chip with gel-stain solution and filtered gel, 6 μL diluted sample and Pro260 ladder were pipetted into each of the sample wells (*1–10* in Figs 2B and 3B); the analysis ran for 5 min.

RESULTS

Out of 1244 *I. ricinus* ticks *Bbl* strains were detected in 321 (25.8 %) ticks by DFM. The 16S rRNA PCR screening detected 371 borrelial amplicons (from 170 females, 13 males and from 188 nymphs and nymphal pools). Sequence analysis of the 16S rRNA and *ospA* amplicons was performed. Forty % (149) of 371 PCR-positive tick samples contained DNA of *B. garinii*, 36.3 % (135) were *B. afzelii*-positive, 12.9 % (48) harbored *Bbs*, 3.5 % (13) were *B. valaisiana*-positive, 0.8 % (3) *B. lusitaniae* positive and 0.5% (2) showed DNA of *B. bissettii* and *B. miyamotoi*-like spirochetes. The rest of 5 % PCR-positive ticks still remain to be identified. Thirty *Borrelia* strains isolated from DFM positive ticks (9.3 %) in BSK-H medium were identified by RT-PCR and by EE chip technology.

The relationship among *ospA* amplicons isolated from ticks and control strains is demonstrated in a phylogenetic tree – very rare *Bbi* E50, *Bbs* strain K4, DNA isolates 21A3, 88A3, 92A3 and 94A3, *B. garinii* strain K32 and *B. valaisiana* strain K75 to the *B. afzelii* control strain 93M are shown in Fig. 1A. Fig. 1B shows the near relation of *B. valaisiana* strain K87 isolated from nymph (Table II) to control strain E5 and

Table II. Different strains of *Borrelia* sp. isolated from *I. ricinus* ticks in different areas identified by RT-PCR and EE chips

Genomic species	Strains from			Area no.[a]
	females	males	nymphs	
B. burgdorferi sensu stricto	KL65, KL76		K42, K44, K76, K4	*1, 4, 10, 12, 13, 14*
B. garinii	KL78, KL81	KL85	K32, K69, K80, K47, K27, K60	*1, 2, 3, 4, 8, 14*
B. afzelii	KL43, KL45 KL67	KL75	K90, K67, K58	*6, 7, 9, 10, 13*
B. valaisiana			K52, K87, K75	*1, 3, 4*
B. bissettii	KL86		K51	*2, 14*
B. lusitaniae			K36	*6*
B. miyamotoi			K91, K47	removed nymphs

[a]*See* Table I.