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Prevalence of *Borrelia burgdorferi* s.l. OspA types in *Ixodes ricinus* ticks from selected localities in Slovakia and Poland

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Abstract

In this study, 746 questing *Ixodes* (*I.*) *ricinus* ticks from eastern Slovakia and 187 ticks from southern Poland were investigated for infection with *Borrelia* (*B.*) *burgdorferi* sensu lato and different outer surface protein A (OspA) types by an improved restriction fragment length polymorphism (RFLP) analysis of the *ospA* gene. The method enables differentiation of both single and multiple infections with *B. burgdorferi* s.s. (OspA type 1), *B. afzelii* (OspA type 2), *B. garinii* (OspA types 3–8), *B. valaisiana* (subgroups I and II), *B. lusitaniae, B. bissettii*, and the recently described genospecies A14S. Broad heterogeneity in *B. burgdorferi* s.l. was found including all species and subtypes except for *B. lusitaniae, B. bissettii,* and genospecies A14S. Regional prevalence of *B. burgdorferi* s.l. varied between 8% and 22.5%. The most frequent species were *B. garinii* (45.4%) and, notably, *B. burgdorferi* s.s. (31.3%). *I. ricinus* nymphs harbored almost exclusively *B. burgdorferi* s.s. and *B. garinii* OspA type 4, while in adults a broad variety of *B. burgdorferi* s.l. types was found. Mixed infections were significantly more often in nymphs than in adult ticks. In all mixed infected nymphs, *B. burgdorferi* s.s. with OspA type 4 was present. These data strongly suggest that *B. burgdorferi* s.s. and *B. garinii* OspA type 4 are maintained in these areas by specific transmission cycles involving a so far undetermined vertebrate host which is frequently fed on by *I. ricinus* larvae. This improved method provides a reliable tool for epidemiological studies on the heterogeneity of *B. burgdorferi* species and OspA types, an important prerequisite for improved local risk assessment and for test- and vaccine development for Europe.

Keywords: *Borrelia burgdorferi*; OspA-subtypes; *Ixodes ricinus* ticks; PCR; Restriction fragment length polymorphism; Lyme borreliosis

Introduction

In recent years, new molecular assays have been developed which improved the direct detection and the classification of *Borrelia burgdorferi* sensu lato strains. Up to now, the *B. burgdorferi* s.l. complex comprises at least 11 species (Wang et al., 1999b; Masuzawa et al., 2001). So far, three of them have been clearly established as pathogenic to humans, namely *B. burgdorferi* sensu stricto [outer surface protein A (OspA) type 1], *B. afzelii* (OspA type 2), and *B. garinii* (OspA types 3–8) (Wilske, 2003). Moreover, *Borrelia* strain A14S, most likely a

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new *Borrelia* species, was recently cultured from human skin biopsy specimens of erythema migrans (EM) in The Netherlands (Wang et al., 1999a).

Borrelia isolates from ticks, reservoir hosts, and humans have been found to be heterogeneous, but some associations between certain *B. burgdorferi* s.l. species, certain hosts, and certain clinical manifestations in patients have already been observed. *B. afzelii* dominates in skin disease (EM, acrodermatitis chronica athropicans [ACA]) of European patients (Wilske et al., 1993, 1996b; Ohlenbusch et al., 1996; Ružić-Sabljić et al., 2000), while *B. garinii* isolates (mostly OspA type 4) are more likely associated with neuroborreliosis (Eiffert et al., 1995; Wilske et al., 1996a). A primarily limited link between *B. burgdorferi* s.s. and Lyme arthritis has been shown to be disputable, as three species, *B. burgdorferi* s.s., *B. afzelii*, and *B. garinii*, have been detected in synovial fluid from patients in Europe (Vasiliu et al., 1998).

Different *B. burgdorferi* s.l. species seem to circulate in different enzootic cycles. In Europe, *B. burgdorferi* s.s. has not been clearly linked with any vertebrate host so far, while *B. afzelii* seems to be associated with rodents, and *B. garinii* and *B. valaisiana* may predominantly be maintained through bird-tick transmission cycles (Humair et al., 1995, 1998, 1999; Olsén et al., 1995; Gern et al., 1997; Kurtenbach et al., 1998, 2001, 2002; Hanincová et al., 2003a, b).

OspA has been shown to be a promising candidate for an effective vaccine for Europe (Gern et al., 1997). Since the present knowledge on the ecology and epidemiology of different *B. burgdorferi* s.l. species and different OspA types is still poor, further information on the distribution of different *B. burgdorferi* s.l. species and subspecies (OspA types) in their natural reservoir hosts and vectors is needed (Eiffert et al., 1995; Wilske et al., 1996a, b; Escudero et al., 2000; Michel et al., 2003). This is an essential prerequisite for a better understanding of borrelia circulation in natural foci on the species and subspecies level as well as for the establishment of effective preventive strategies against Lyme borreliosis.

Although there are some data on the distribution of *B. burgdorferi* s.l. species in Slovakia and Poland, no studies regarding the heterogeneity of OspA types are available. Thus, the aim of the present study was to define the prevalence of different *B. burgdorferi* s.l. species and OspA types in *Ixodes ricinus* ticks in selected areas in Slovakia and Poland.

Material and methods

Field sites and tick sampling

I. ricinus ticks (nymphs and adults) were collected by flagging the vegetation in oak-hornbeam (Carpineto-Quercetum) deciduous forest at three localities in the Košice region in eastern Slovakia which are about 30 km distant from each other (Furča: March to October in 2001, 2002, and 2003; Rozhanovce: March to October in 2001 and 2002; and Malá Ida: September 2001) and in the Tarnow region in southern Poland (May 2002) about 150 km north of Košice. All regions belong to the western Carpathian curving along the borders of the Czech Republic, Slovakia, Poland, Austria, and Hungary. Collected ticks were immediately stored in 1.5 ml collection tubes filled with 70% ethanol until use. Each tick was precisely determined to species and life stage by an entomologist and only *I. ricinus* ticks were analyzed further.

DNA extraction from ticks

Single ticks were air-dried and crushed in individual 1.5 ml microcentrifuge tubes in 200 µl phosphate-buffered saline (PBS, pH = 7.2) using a separate sterile laboratory spatula. DNA was extracted with High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. The final volume of DNA was eluted in 80 µl. As extraction negative control one 1.5 ml tube with PBS was processed in parallel for each 10 tick extractions. The samples were either directly used for PCR or stored at −20 °C until use.

PCR amplification

The PCR targeting the *ospA* gene was essentially done as described previously (Michel et al., 2003). The amplification was carried out in a thermal cycler Gene Amp PCR System 2700 (Applied Biosystems, Foster City, Canada). An aliquot (5 µl) of the extracted DNA was added to the master mix containing 200 mM of each nucleotide (Roche, Mannheim, Germany), 5 µl of 10 × buffer (Roche, Mannheim, Germany), 10 pmol of each primer, V1a, V1b, R2, and R37 (Metabion, Martinsried, Germany) (Table 1), 29.5 µl distilled water, and 0.5 U Taq polymerase (Roche, Mannheim, Germany). DNA was initially denaturized at 95 °C for 5 min, followed by 30 cycles of 45 s denaturation at 94 °C, 45 s annealing at 50 °C, and 1 min extension at 72 °C. The PCR was completed by a final extension for 7 min at 72 °C. A 5 µl volume from the first step was subjected to semi-nested amplification using primers V3a, V3b, R2, and R37 (Table 1) using the same conditions as for the first step. Amplification products (5 µl) were visualized on a 2% agarose gel, stained with ethidium bromide (1%), and documented with a Gel Documentation System (Biorad, Munich, Germany). Genomic DNA corresponding to five borreliae per PCR reaction of strains PKa2 (*B. burgdorferi* s.s., OspA type 1), PKo (*B. afzelii*, OspA type 2), and PBi (*B. garinii*, OspA type 4) served as positive controls. Negative controls contained distilled water instead of the extracted DNA. All samples were checked for inhibitory substances by β-actin PCR (inhibition control) (Murray et al., 1990).

Restriction fragment length polymorphism (RFLP) analysis

The template DNA of positive samples was separately digested with the restriction enzymes *Ssp*I, *Sfu*I, *Bgl*II, *Kpn*21,

Table 1. Oligonucleotides for amplification of the *ospA* gene of *Borrelia burgdorferi* s.l

Primer	Amplification	Sequence	Position
V1a (forward)	Primary	5′-GGG AAT AGG TCT AAT ATT AGC-3′	18–38
V1b (forward)	Primary	5′-GGG GAT AGG TCT AAT ATT AGC-3′	18–38
V3a (forward)	Nested	5′-GCC TTA ATA GCA TGT AAG C-3′	37–55
V3b (forward)	Nested	5′-GCC TTA ATA GCA TGC AAG C-3′	37–55
R2 (reverse)	Both	5′-CAT AAA TTC TCC TTA TTT TAA AGC-3′	832–855
R37 (reverse)	Both	5′-CCT TAT TTT AAA GCG GC-3′	829–845

Table 2. Predicted RFLP patterns for different *Borrelia burgdorferi* s.l. species and OspA types (modified after Michel et al. (2003))

Strain	Species	OspA type	Predicted RFLP pattern (bp)					
			*Ssp*I	*SfuI*	BglII	*Kpn*21	*Hind*III	*Xba*I
PKa2	*B. burgdorferi* s.s.	1	534/264	798	798	798	654/144	[a]
CA 8	*B. burgdorferi* s.s.	1	534/264	798	798	429/369	654/144	[a]
PKo	*B. afzelii*	2	798	537/261	798	798	798	[a]
PBr	*B. garinii*	3	801	801	758/43	429/372	801	[a]
PBi	*B. garinii*	4	798	798	556/242	798	798	[a]
PHei	*B. garinii*	5	798	798	798	549/195/54	654/144	[a]
TN	*B. garinii*	6	801	801	801	429/252/177	585/144/72	606/122/73
PRef	*B. garinii*	7	801	801	758/43	428/372	657/144	[a]
PKi	*B. garinii*	8	801	801	801	429/252/177	585/144/72	725/76
VS116	*B. valaisiana* subgroup I	—	801	801	801	801	465/336	798
NE231	*B. valaisiana* subgroup II	—	798	798	665/133	798	665/133	556/242
A14S	*Borrelia* A14S	—	798	798	665/133	798	665/133	798

[a]Not tested in this study.

and *Hind*III (Roche, Mannheim, Germany) (Michel et al., 2003). Samples that showed restriction patterns compatible with *B. burgdorferi* s.l. OspA type 6, OspA type 8, *B. valaisiana* subgroup II, or *Borrelia* genospecies A14S were additionally digested with the restriction enzyme *Xba*I (Table 2), which enables differentiation of OspA type 6 from OspA type 8 (Wilske et al., 1996a) as well as *B. valaisiana* subgroup II from genospecies A14S (Wang et al., 1997, 1999a). Restriction fragments were electrophoresed, visualized, and documented as described for amplification products.

Cloning and sequencing

An aliquot of the cleaned PCR amplicon was ligated with the pCRII vector of TA Cloning® Kit (Invitrogen, Carlsbad, USA) overnight at 10 °C according to the manufacturer's instructions. Ligands were transformed in competent One Shot® INVαF′ *Escherichia coli* cells and plated on LB-amp/IPTG/X-Gal medium (ampicillin 5 μg/ml, IPTG 0.2 mM, X-Gal 20 μg/ml). The optimal transformants were screened for their inserts by PCR using primers NEB 1233 and Plaz-41. Vectors with the target DNA fragment were isolated according to the protocol of High Pure Plasmid Isolation Kit (Roche, Mannheim, Germany). Sequencing was performed by Agowa Sequencing Services (Berlin, Germany). *ospA* sequences were processed using the program DNAMAN Version 5.2.9 (Lynnon Biosoft, Vaudreuil, Canada) and CHROMAS Version 1.45 (Technelysium, Helensvale, Australia) and compared to homologous DNA fragments in the GenBank database.

Statistical analysis

Differences in the prevalence of *B. burgdorferi* s.l. in *I. ricinus* nymphs versus adults were evaluated statistically using the two-tailed χ^2- test (degrees of freedom, df = 1). A *p*-value of $\leqslant 0.05$ was considered statistically significant.

Results

Improvement of RFLP

Re-evaluation of the RFLP with all *B. burgdorferi* s.l species and subspecies revealed that according to the previous RFLP protocol (*Ssp*I, *Sfu*I, *Bgl*II, *Kpn*21, and *Hind*III digestion) we were not able to distinguish OspA type 6 from OspA type 8 and *B. valaisiana* subgroup II from genospecies A14S (Table 2, Fig. 1). Therefore, we analyzed the *ospA* sequences of these strains and searched in a complex of restriction enzymes for a suitable one. Finally, the *Xba*I restriction enzyme was found to be a reliable tool for the differentiation of single infections with the above-mentioned strains.

Solely a triple infection comprising *B. valaisiana* subgroup I, *B. valaisiana* subgroup II, and genospecies A14S cannot be distinguished from a *B. valaisiana* subgroup I/II double infection due to the overlapping restriction patterns (Fig. 1, Table 2).

Subcloning and sequencing

Two restriction profiles were assessed as disputable. Subcloning and *ospA* sequencing revealed one triple infection with *B. valaisiana* subgroup I/II, and *B. garinii* OspA type 7 in a female tick from Furča, and one double infection with *B. valaisiana* subgroup I/II in a male tick from Malá Ida, both from Slovakia (Fig. 1).

Prevalence of *Borrelia burgdorferi* s.l. in *Ixodes ricinus* ticks

The results of semi-nested PCR amplification showed that in total 138 of 933 ticks (96 of 746 ticks from

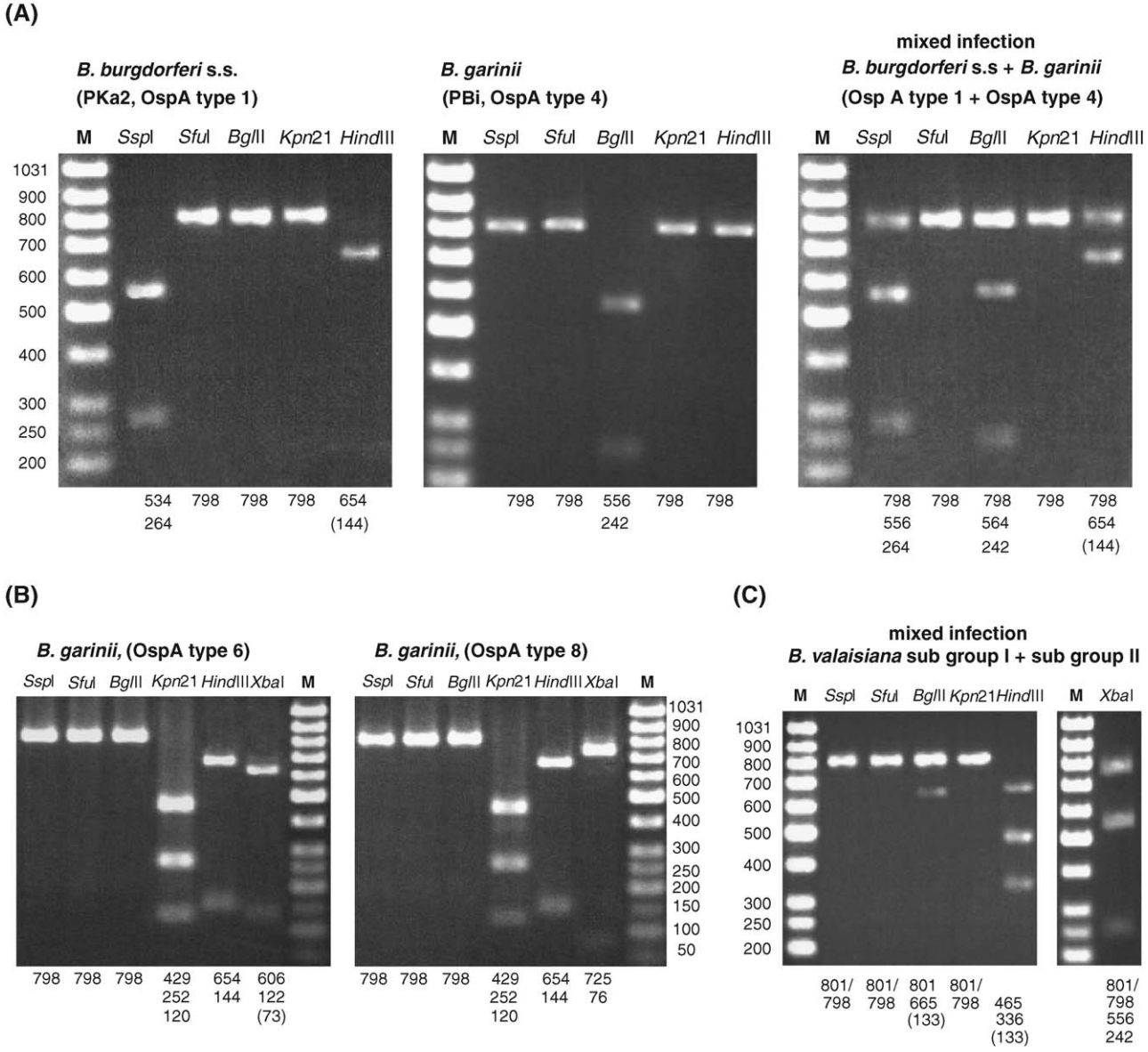

Fig. 1. Examples of RFLP patterns. (A) *Borrelia burgdorferi* s.s. OspA type 1/*B. garinii* OspA type 4; single and mixed profiles. (B) *B. garinii* OspA type 6 and OspA type 8. *Note*: Only *Xba*I digestion enables differentiation of OspA type 6 from OspA type 8. (C) Mixed infection: *B. valaisiana* subgroup I/subgroup II. *Note*: This restriction profile is identical with that predicted for a triple infection of *B. valaisiana* subgroup I/subgroup II/genospecies A14S. Differentiation was done by subcloning and sequencing. The respective restriction enzymes are shown on the top. The predicted size of bands after restriction is listed below. Bands in parentheses were not visible on a gel. M: molecular weight marker (GenRuler™ 50 bp DNA Ladder, Fermentas, Germany); predicted band sizes of 801 and 798 bp have not been distinguishable.

Slovakian areas and 42 of 187 ticks from the Polish region) were infected with *B. burgdorferi* s.l. (Table 3). Local prevalence ranged from 8% in Rozhanovce in 2002 to 22.5% for the Tarnow region in 2002 (Malá Ida was not considered in 2001 due to the small number of investigated ticks). Altogether, nymphs in Slovakia had a significantly lower infection rate than adults ($p \leqslant 0.001$), while the prevalences in nymphs and adults in Poland did not differ significantly (Table 3).

Differentiation of *Borrelia burgdorferi* s.l. species and OspA types by *ospA* PCR/RFLP-based analysis

Based on restriction pattern analysis of the *ospA* gene, all species and subtypes were present except for *B. lusitaniae, B. bissettii,* and genospecies A14S (Fig. 2, Table 4). Overall, *B. garinii* (45.4%) was the predominant species, followed by *B. burgdorferi* s.s. (31.3%), *B. afzelii* (12.3%), and *B. valaisiana* (11.1%). Within *B. garinii*, most prevalent were the types 4 and 6 which accounted for 48.6% and 21.6% of all *B. garinii* OspA types, respectively. In addition, both *B. valaisiana* subgroups as described by Wang et al. (1997) were detected and could even be differentiated in mixed infections in single ticks. When considering the prevalence of different *Borrelia* species and subtypes in different tick stages, a different picture emerges (Fig. 2): In unfed adults, all species and subtypes were present but *B. garinii* was significantly more frequent than *B. afzelii*, *B valaisiana*, or *B. burgdorferi* s.s. In contrast, the clearly predominant species in unfed nymphs was *B. burgdorferi* s.s. (61%) followed by *B. garinii* OspA type 4 (28.8%), while the other species and subtypes were rare or not detected at all. Significant differences ($p \leqslant 0.01$) were found for all species and for *B. garinii* OspA type 6 when comparing the prevalence in nymphs versus adults.

With regard to the different study sites of nymphal ticks, a uniform pattern was observed in all locations (Malá Ida: no nymphs investigated): predominance of

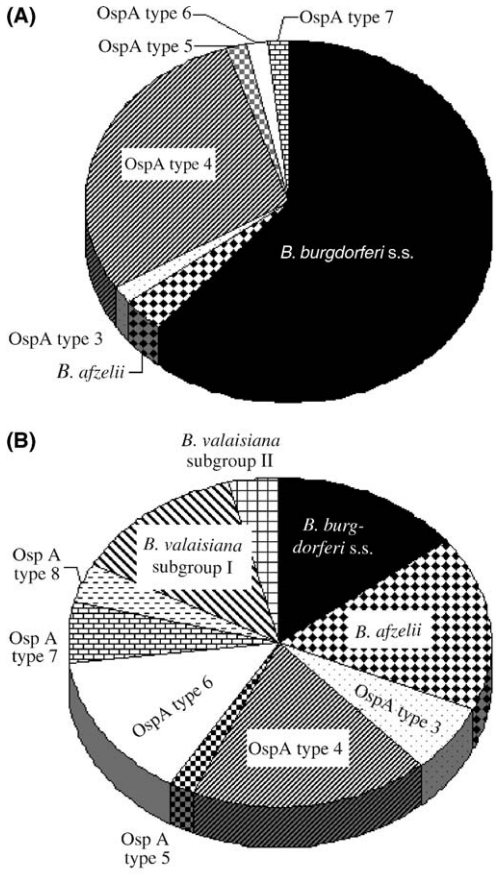

Fig. 2. Tick stage-related prevalence of *Borrelia burgdorferi* s.l. species and subtypes. (A) Prevalence of *B. burgdorferi* s.l. species and OspA types in *Ixodes ricinus* nymphs ($n = 59$ types). (B) Prevalence of *B. burgdorferi* s.l. species and OspA types in *I. ricinus* adults ($n = 104$ types). *Note*: Mixed infections are included in the numbers.

Table 3. Prevalence of *Borrelia burgdorferi* s.l. in *Ixodes ricinus* ticks from Slovakia and Poland

	Nymphs		Adults		Total	
	Examined	Positive (%)	Examined	Positive (%)	Examined	Positive (%)
Slovakia						
Furča 2001	53	7 (13)	73	12 (16)	126	19 (15)
Furča 2002	33	2 (6)	109	20 (18)	142	22 (15)
Furča 2003	97	1 (1)	116	27 (23)	213	28 (13)
Rozhanovce 2001	144	12 (8)	4	0 (0)	148	12 (8)
Rozhanovce 2002	67	6 (9)	21	1 (5)	88	7 (8)
Malá Ida 2001	—	—	29	8 (28)	29	8 (28)
Slovakia total	394	28 (7)	202	68 (19)	746	96 (13)
Poland						
Tarnow region 2002	83	18 (22)	104	24 (23)	187	42 (22)

Table 4. Prevalence of *Borrelia burgdorferi* s.l. species and OspA types in *Ixodes ricinus* ticks at selected localities in Slovakia and Poland

Locality		Slovakia													Poland				
		Furča						Rozhanovce				Malá Ida	Total		Tarnow		Total		
Year		2001[a]		2002[b]		2003[c]		2001[d]		2002[e]		2001[f]			2002[g]				
No. of ticks		53	73	33	109	97	116	144	4	67	21	29	394	352	83	104	477	456	933
Species	OspA type	N (%)[h]	A (%)[h]	N (%)[h]	A (%)[h]	N (%)[h]	A (%)[h]	N (%)[h]	A (%)[h]	N (%)[h]	A (%)[h]	A (%)[h]	N (%)[h]	A (%)[h]	N (%)[h]	A (%)[h]	N (%)[h]	A (%)[h]	N+A (%)[h]
BB. s.s.	1	4 (44)	5 (42)	2 (67)	3 (13)		3 (9)	10 (71)		6 (55)	1 (100)		22 (58)	12 (15)	14 (67)	3 (12)	36 (61)	15 (14)	51 (31)
B. afzelii	2		3 (25)		2 (9)		1 (3)			1 (9)		5 (50)	1 (3)	11 (14)	1 (5)	7 (28)	2 (3)	18 (17)	20 (12)
B. garinii	3				1 (4)	1 (100)	6 (18)						1 (3)	7 (9)			1 (2)	7 (7)	8 (5)
	4	4 (44)	1 (8)	1 (33)	1 (4)		14 (42)	2 (14)		4 (36)			11 (29)	16 (20)	6 (29)	3 (12)	17 (29)	19 (18)	36 (22)
	5		1 (8)				1 (3)	1 (7)					1 (3)	2 (3)			1 (2)	2 (2)	3 (2)
	6		1 (8)		5 (22)		2 (6)	1 (7)					1 (3)	8 (10)		7 (28)	1 (2)	15 (14)	16 (10)
	7	1 (11)			2 (9)		3 (9)					1 (10)	1 (3)	6 (8)			1 (2)	6 (6)	7 (4)
	8				1 (4)		3 (9)						0 (0)	4 (5)			0 (0)	4 (4)	4 (2)
B. garinii	3–8	5 (56)	3 (25)	1 (33)	10 (43)	1 (100)	29 (88)	4 (29)	0 (0)	4 (36)	0 (0)	1 (10)	15 (39)	43 (54)	6 (29)	10 (48)	21 (36)	53 (51)	74 (45)
Bv. sub. I	—		1 (8)		5 (22)							3 (30)	0 (0)	9 (11)		5 (20)	0 (0)	14 (13)	14 (9)
*Bv.*sub. II	—				3 (13)							1 (10)	0 (0)	4 (5)			0 (0)	4 (4)	4 (2)
Total[i]		9	12	3	23	1	33	14	0	11	1	10	38	79	21	25	59	104	163

BB. s.s.: *B. burgdorferi* sensu stricto; *Bv.* sub. I (II): *B. valaisiana* subgroup I (II); N: nymphs; A: adults.

[a]OspA types 1+4 (2 nymphs).

[b]OspA types 1+4 (1 nymph), *B. valaisiana* subgroup I+II (1 male), OspA types 6+7+8 (1 female).

[c]OspA types 1+4 (3 males), OspA types 3+4 (1 male), OspA types 3+6 (2 females).

[d]OspA types 1+4 (2 nymphs).

[e]OspA types 1+4 (3 nymphs), OspA types 1+2+4 (1 nymph).

[f]OspA type 7+*B. valaisiana* subgroup I+II (1 female).

[g]OspA types 1+4 (3 nymphs), OspA types 1+6 (1 female).

[h]Percentage of OspA type (in relation to the number of positive nymphs and/or adults).

[i]Mixed infections are included in the numbers.

B. burgdorferi s.s. followed by *B. garinii* OspA type 4. In adults, however, different patterns were found (Fig. 3): In Furča, all species and subtypes found in this study were present in adults, but *B. garinii* predominated (61.8%) and thereof OspA type 4 was most common. In Malá Ida, *B. afzelii* and *B. valaisiana* dominated, and in Rozhanovce only one *B. burgdorferi* s.s. infected adult was found. In the Polish area of Tarnow, *B. garinii* was most common but also *B. afzelii*, *B. valaisiana*, and *B. burgdorferi* s.s. were frequent.

When comparing the different years of tick collection (Tables 3 and 4) in Furča, the prevalence of *B. burgdorferi* s.l. in nymphs decreased significantly ($p<0.05$) from 2001 to 2003, while the prevalence in adults tended to increase ($p>0.05$) at the same time. In Rozhanovce (only nymphs considered), a trend for a higher prevalence was observed in 2002 when compared to 2001. With regard to the abundance of different species and subtypes, the only statistically significant change was the increased prevalence of *B. garinii* OspA type 4 in adult *I. ricinus* ($p<0.02$) in Furča in 2003. Notable but not significant findings in Furča comprise the high prevalence of both *B. valaisiana* subgroups in adults in 2002 and a decreasing prevalence of OspA type 4 in nymphs.

Mixed infections were found in 18 ticks (18.8% of the positive ticks) in Slovakia and four samples (9.5%) in Poland (Table 5). At least one mixed infection was detected at each study site. The prevalence of mixed infections in nymphs (26.1% of all positive nymphs) was significantly higher ($p\leqslant 0.025$) than in adults (10.9% of all positive adults). Both in Košice and Tarnow regions, the combination *B. burgdorferi* s.s./*B. garinii* OspA type 4, the two predominant OspA types at the study locations, was most common ($n=14$, Table 5). In four ticks, mixed infections comprised solely different *B. garinii* OspA types: two times OspA type 3/6, and in one tick each OspA type 3/4 and 6/7/8. One tick each was infected with *B. burgdorferi* s.s./OspA type 6, *B. burgdorferi* s.s./*B. afzelii*/OspA type 4, *B. valaisiana* subgroup I/subgroup II, and *B. valaisiana* subgroup I/subgroup II/OspA type 7.

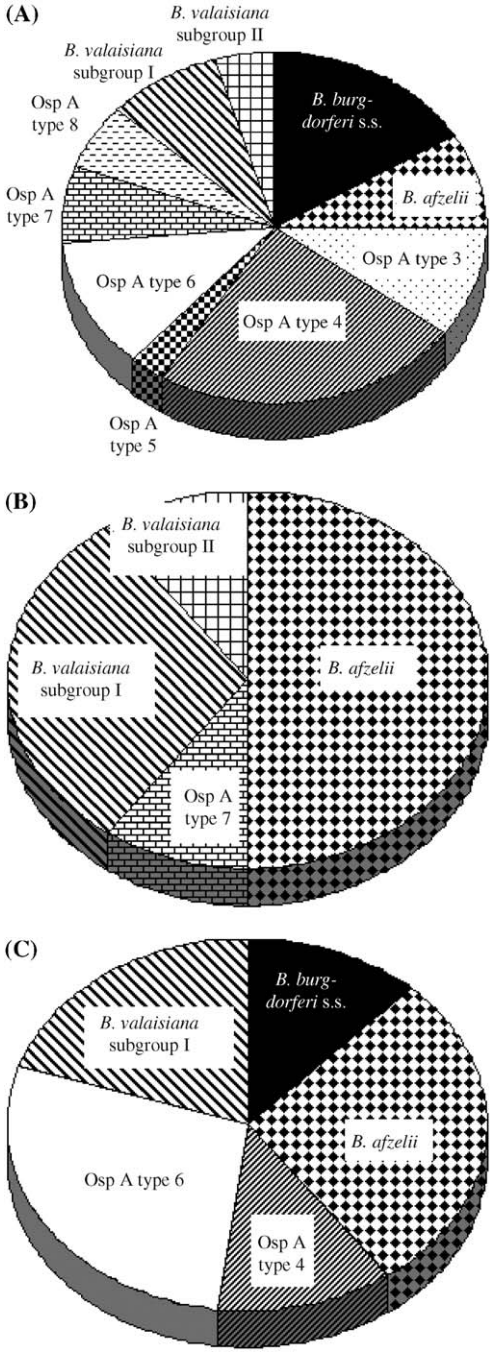

Fig. 3. Regional prevalence of *Borrelia burgdorferi* s.l. species and subtypes in adult *Ixodes ricinus* ticks. (A) Locality Furča: $n=68$ ticks positive; (B) locality Malá Ida: $n=10$ ticks positive; (C) locality Tarnow: $n=25$ ticks positive. *Note*: Mixed infections are included in the numbers.

Discussion

Since different species and OspA types of *B. burgdorferi* s.l. obviously have a different pathogenic potential, information on their distribution in tick populations is a basic requirement for local risk assessment, for the development of diagnostic tests as well as vaccines based on OspA. The present study provides for the first time data on the distribution of *B. garinii* OspA types and *B. valaisiana* subgroups from selected areas in Slovakia and Poland using an improved *ospA*-based RFLP analysis. Moreover, we present evidence that *B. burgdorferi* s.s. and *B. garinii* OspA type 4 seem to circulate in a specific cycle in these areas.

Table 5. Mixed *Borrelia* infections in *Ixodes ricinus* ticks according to tick stage

	Double infections				Triple infections				Total
Mixed infections	OspA type 1+4	OspA type 1+6	OspA type 3+4	OspA type 3+6	*Bv.* sub. I+II	OspA type 1+2+4	OspA type 6+7+8	OspA type 7+ *Bv.* sub. I+II	
Nymphs	11					1			12
Adults	3	1	1	2	1		1	1	10
	14	1	1	2	1	1	1	1	22

Bv. sub.: *B. valaisiana* subgroup(s).

Improvement of RFLP analysis

In the present study, we applied an *ospA* PCR-based RFLP analysis, which has been shown to be a sensitive and effective technique for the detection of single and mixed infections of different *B. burgdorferi* s.l. species and OspA types (Michel et al., 2003). It has been shown that this PCR does not preferentially amplify certain species or *ospA* types, and even mixed DNA of cultured borreliae could be reliably differentiated (Michel et al., 2003). A relevant improvement of this method was achieved by adding the *Xba*I enzyme for RFLP analysis. In this way, it is possible to differentiate OspA type 8 from OspA type 6 as well as *B. valaisiana* subgroup II from genospecies A14S. However, it became clear during the study that also this method has some limitation: Due to overlapping restriction profiles (Table 2, Fig. 1C), it is impossible to differentiate a *B. valaisiana* subgroup I/subgroup II double infection of a single tick from a *B. valaisiana* subgroup I/subgroup II/genospecies A14S triple infection. Such a restriction profile was present in two of the studied ticks. In one case, a *B. valaisiana* subgroup I/subgroup II double infection, in the other tick, a triple infection with *B. valaisiana* subgroup I/subgroup II/*B. garinii* OspA type 7 could be verified by additional subcloning and sequencing. However, this kind of procedure cannot completely rule out an additional infection with the genospecies A14S in these ticks. This indicates that the precise differentiation of different *Borrelia* subspecies especially in mixed infections is more sophisticated than primarily supposed.

Prevalence of *Borrelia burgdorferi* s.l. in *Ixodes ricinus* ticks

Our results are in line with the overall prevalence of *B. burgdorferi* s.l. in host-seeking ticks in Europe, which varies from approximately 1.9% (0–11%) in unfed larvae, approximately 10.8% (2–43%) in unfed nymphs to approximately 17.4% (3–58%) in unfed adults (Hubálek and Halouzka, 1998). Studies from Poland and Slovakia are also in accordance with our results: In a study on 2818 *I. ricinus* collected in 1994–1997 in eastern Slovakia (Košice district), *B. burgdorferi* s.l. prevalence was 4.8–17.2% and, like in our study, nymphs had a lower infection rate than adults (Štepánová-Tresová et al., 2000). The overall *B. burgdorferi* s.l. prevalence in ticks from two localities in southern Poland was 15.5% and 37.5% (Stańczak et al., 2000).

Prevalence of *Borrelia burgdorferi* s.l. species and OspA types

The prevalence of different *Borrelia* species may differ considerably between European countries and even

between closely located areas. Numerous studies recognized *B. garinii* or *B. afzelii* as the predominant species in most European countries, and the prevalence of the mostly rare species *B. burgdorferi* s.s. seems to decrease from west to east (e.g. Rijpkema et al., 1996; Tresová et al., 1998; Gern et al., 1999; Stańczak et al., 2000; Derdáková et al., 2003; Hildebrandt et al., 2003; Jouda et al., 2003; Michel et al., 2003). Accordingly, *B. garinii* was, altogether, the predominant species in the present study but, surprisingly, followed by *B. burgdorferi* s.s. This is in sharp contrast to the above-mentioned studies, especially to a study recently conducted in western Slovakia, where less than 2% of 420 infected ticks harbored *B. burgdorferi* s.s., and *B. afzelii* was by far the most prevalent species (Hanincová et al., 2003b). Moreover, we found considerable year-to-year variations within particular regions and also considerable variation in the prevalence of different *B. burgdorferi* s.l. species and subtypes among different regions and different life stages of ticks. These findings underscore that the prevalence of *B. burgdorferi* s.l. species and subtypes may even vary substantially between closely located areas.

Several authors suggested that certain species of the *B. burgdorferi* s.l. complex seem to be associated with certain vertebrate hosts. *B. afzelii* and some *B. garinii* strains appear to use rodents as main reservoirs, while *B. valaisiana* and most *B. garinii* strains were found in enzootic cycles with birds as main vertebrate hosts (Humair et al., 1995, 1999; Olsén et al., 1995; Gern et al., 1998; Huegli et al., 2002; Kurtenbach et al., 1998, 2001, 2002). Furthermore, it was shown that these associations are mirrored in the complement sensitivity of these species or strains, i.e., rodent-associated strains are resistant to rodent complement but sensitive to bird complement and vice versa. It was therefore proposed that the complement is a key determinant for the host preference of different *B. burgdorferi* s.l. species (Kurtenbach et al., 2002). For *B. burgdorferi* s.s., to our knowledge such a specific cycle has not been described for Europe, so far. *B. burgdorferi* s.s. displays partial resistance to avian and mammalian complement, and it was shown that *B. afzelii* is distinctly more efficiently transmitted from *Apodemus* mice to ticks than *B. burgdorferi* s.s. (Kurtenbach et al., 2002; Richter et al., 2004), the latter of which might be more a generalist.

A more detailed analysis of our data set also provides clues for such cycles. *B. burgdorferi* s.s. surprisingly was the clearly predominant species in questing *I. ricinus* nymphs accounting for more than 60% of all strains found while this species accounted for only 14.4% in adult *I. ricinus*. Furthermore, *B. garinii* OspA type 4 was the second most frequent strain in unfed nymphal ticks, where these two types together accounted for about 90% of all infections. This exceptional pattern was uniformly observed at all collecting sites where a sufficient number of nymphs was investigated. Notably, a broad variety of *Borrelia* species and subspecies was present in adult ticks, showing that almost all relevant *B. burgdorferi* s.l. OspA types described for Europe co-circulate in these areas. The pattern found in nymphs therefore cannot be explained by a selective local prevalence of only these two types. These findings together led us to the assumption that specific transmission cycles must exist involving larval and nymphal *I. ricinus*, *B. burgdorferi* s.s., and *B. garinii* OspA type 4, and (a) so far undetermined vertebrate host(s). Moreover, the pattern found for mixed infections in nymphs may argue for a single host species which is a reservoir host for both types, *B. burgdorferi* s.s. and *B. garinii* OspA type 4. *I. ricinus* ticks may acquire a borrelia infection during the blood meal on an infected host and usually transmit the infection transstadially but only occasionally transovarially (Monin et al., 1989). Therefore, mixed infections may result from feeding on different infected hosts or feeding on one host infected with multiple *B. burgdorferi* s.l. types at the same time. Furthermore, co-feeding transmission, i.e., transferring infections directly from one tick to another while feeding together on the same host individual, may result in multiple infections in single ticks (Gern and Rais, 1996). Since questing nymphs had only fed once as larvae on a single host and transovarial transmission is rare, mixed infections in unfed nymphs either result from a host infected with the respective borrelia types or through co-feeding transmission. Therefore, one or several host species must be present in these areas that serve as main host(s) for *I. ricinus* larvae and act as a kind of filter in favor of *B. burgdorferi* s.s. and *B. garinii* OspA type 4.

Overall, a striking variety of *B. burgdorferi* species and OspA types was found at the study sites, indicating that in terms of test and vaccine development for Europe, none of these species or OspA types can be neglected. Furthermore, we found strong evidence for a further transmission cycle which specifically favors *B. garinii* OspA type 4 and *B. burgdorferi* s.s. which is rather rare in Europe.

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