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HETEROGENEITY OF *BORRELIA BURGDORFERI* SENSU LATO AND THEIR REFLECTION ON IMMUNE RESPONSE

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Abstract: Geographically different strains of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* sensu stricto Ir 105, *B. burgdorferi* s.s. + *B. afzelii* V 123, *B. garinii* Ir 112 - isolates from eastern Slovakia, *B. garinii* K24 - isolate from western Slovakia and *B. burgdorferi* s.s. B 31 - American strain) were compared as antigens for serological study of Lyme borreliosis by IgG ELISA on a group of horses from eastern Slovakia. In a set of 101 horse serum samples, positivity with the use of Ir 105 strain was 53 (52.4%), with V 123 51 (51.49%), with Ir 112 48 (47.5%), with K 24 47 (46.5%) and with B 31 only 25 (24.7%). The seroprevalence between strains B 31 and Ir 105, B 31 and V 123, B31 and Ir112, B 31 and K 24 differed statistically significantly (test χ^2, $p<0.05$); however, the differences between strains Ir 105, V 123, Ir 112 and K 24 were insignificant. Consistency of positive and negative findings between American and Slovak strains ranged from 50.5–62.4%. Comparison of Slovak strains (Ir 105, V 123, Ir 112 and K 24) consistency of positive and negative findings was higher from 79.2–95.04% The highest consistency of findings was reached comparing strains Ir 112 and K 24, and the same high agreement of results was observed between the strains Ir 105 and V 123 and also Ir 112 and Ir 105. Higher consistency of findings of serologically examined horses with geographically close strains is in accordance with greater similarity of protein profiles of Slovak strains compared to the American strain.

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INTRODUCTION

Lyme borreliosis is a polysystemic zoonotic disease, evoked by spirochete *Borrelia burgdorferi*. It is transmitted by ticks [2]. Although dogs are very important from the epidemiological point of view [10], the literature also records the occurrence of this zoonosis in free-living animals [18] and farm animals [19], including horses [35, 28]. Diagnosis of Lyme borreliosis is based on epidemiological anamnesis, clinical symptoms, laboratory tests and prompt antibiotic response [3, 29]. Diagnosis of this disease is difficult, particularly in horses, because their anatomy and use in sports predisposes them to various musculoskeletal disorders such as Lyme

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borreliosis that causes limping. Therefore, suspect diagnosis requires elimination of other causes of limping (by radiography, special neurological and musculoskeletal examinations). In view of the frequent asymptomatic course of the disease, its non-specific clinical symptoms and problematic use of direct diagnostic methods [9, 12], the diagnosis is based on indirect diagnostic methods, with ELISA and IFA being the most frequently used [38]. The Western blot method is also being employed more often [7], but is still difficult to standardize and show great interassay variation [16, 17]. Serological tests for Lyme disease have not been standardized until now which is reflected in the noticeable differences of results among laboratories. The heterogeneity of *Borrelia burgdorferi* s.l. strains is one of the intricate problems in serological diagnosis of Lyme borreliosis. The heterogeneity is particularly significant in European strains [16, 17, 46].

The aim of this study was to compare 5 *Borrelia* strains as antigens by performing ELISA in a group of horses from eastern Slovakia. Four of the strains were isolated from the ticks *Ixodes ricinus* in Slovakia and 1 from the American ticks *Ixodes scapularis*.

MATERIALS AND METHODS

Sera. During the years 2000–2002, 101 serum samples were examined for the presence of anti-*Borrelia* IgG antibodies taken from clinically healthy horses from eastern Slovakia. The sera were provided by the State Veterinary Institute in Košice, the veterinary outdoor department of the First Internal Clinic in Košice, the State Veterinary Institute in Prešov and the military training area in Poprad. Specifically, horse sera were examined (race horses, transport of horses between breeding farms, sale, purchase) for the presence of *Malleous*, *Brucellosis*, *Equine Coitus Exanthema*, *Infectious Anemia* and *Rhinopneumonitis* antibodies.

Antigens. The following whole cells sonicated cultures from *Borrelia burgdorferi* sensu lato (s.l.) were used as antigens: *Borrelia burgdorferi* sensu stricto (s.s.) Ir 105, *B. garinii* Ir 112 - our own local strains isolated from *I. ricinus* from the urban area of Košice, *B. burgdorferi* s.s. with a small amount of *B. afzelii* V 123 - isolated from *I. ricinus* from Vihorlat Mountains locality - also from eastern Slovakia, *B. garinii* K 24 - isolated from *I. ricinus* from western Slovakia [32] and *B. burgdorferi* s.s. B 31 - American strain from *I. scapularis* [20]. The sonicated antigens of Ir 105, Ir 112 and V 123 strains were prepared by method of Tresová *et al.* [43]. Electrophoresis and immunoblotting with monoclonal antibodies were used for the identification of the strains Štěpánová-Tresová *et al.* [41]. Antigens from the strains B 31 and K 24 were obtained by a generous donation from the Institute for Research of Rheumatic Diseases in Piešťany. The protein content was measured by the method of Lowry *et al.* [25]. The working dilution of antigens and conjugates was estimated by the box titration.

SDS-PAGE. The sonicated antigens and low-range-molecular-mass standard (BioRad) were dissolved in a sample buffer (containing β-mercaptoethanol as a reducing agent), boiled for 5 min and subjected to SDS-PAGE (12% polyacrylamide gel and 4% acrylamide stacking gel) using the system of Laemmli [24]. Electrophoresis was carried out at a constant current of 25 mA. Coomassie brilliant blue G 250 (Sigma) was used to stain the proteins in the gels. Proteins separated in the other gel were then transferred to a nitrocellulose membrane using the system of Towbin *et al.* [42]. Statistical evaluation was carried out by χ^2 test and coefficient of variation (V%) [37]. Sensitivity and specificity were calculated by Margolis [31].

ELISA. Sera were examined by modified ELISA, as described by Štefančíková *et al.* [40]. Briefly: Microplates were filled in parallel with 100 µl of respective antigen diluted in carbonate buffer at pH 9.6 (B 31- 4.2 µg/ml; K 24 - 3 µg/ml; Ir 105 - 1.5 µg/ml; Ir 112 and V 123 each of 2.5 µg/ml) and incubated overnight at 4°C. After washing 3 times with phosphatate buffer pH 7.2 100 µl portions of sera diluted at 1:400 in phosphatate buffer with 0.05% Tween 20 and 1% BSA, and *Treponema phagedensis* standard lyophilized extract (FTA sorbent) (IMUNA) were added to each well and incubated at 37ºC for 30 min. After a triple washing of the plates, 100 µl portions of anti - horse IgG peroxidase conjugate (Sigma) were added to each well, diluted at 1:2000 for B 31 and 1:3000 for K 24, Ir 105, Ir 112 and V 123. After 30 min of incubation and a subsequent washing, 100 µl of substrate solution (pH 5.0) with orthophenylene diamine was added per well. The reaction was stopped with 5% H_2SO_4 after 15 min of incubation. Absorbance was measured at a wavelength of 492 nm. Positive controls were seropositive sera of horses obtained from the area of Košice, where infected *I. ricinus* had been captured [36], and cases of Lyme disease in humans occur (Reports of the State Health Institute). The negative controls (E_{492}<0.4) were selected from negative samples of 15 horses living in the area where *I. ricinus* occurs very rarely. Cut-off was determined as a value 3 standard deviations above the mean optical density (OD) for negative serum samples.

Interassay reproducibility of ELISA: Parallel with each strain, 20 sera were monitored daily of which 10 were positive and 10 negative. All the sera were repeatedly examined with the lapse of 2 month.

Cross-reacting detection. To take into account the possibility of known cross-reactions to occur in some other serological tests between *Borrelia* and *Leptospira*, we also examined serum samples for the presence of *Leptospira* antibodies. Eight serovars (*L. icterohaemorr-hagiae Petrík*, *L. grippotyphosa Kúty*, *Turňa* , *L. sejroe Šaca*, *L. canicola S-397*, *L. pomona Šimon*, *L. tarassovi S-31*, *L. bataviae Moldava* and *L. ballum Castellon 3*) were used for sera examination using microscopic agglutination

reaction (MAR) [21]. Agglutination reactions at dilutions > 1:100 were considered positive for antibodies Leptospira.

RESULT

Of 101 serum samples from horses examined for the presence of anti-*Borelia* IgG antibodies (Tab. 1), 53 (52.4%) were positive with Ir 105 strain, 47 (46.5%) with K 24 strain, 48 (47.5%) with Ir 112, 51 (51.49%) with V 123 strain and only 25 (24.7%) with B 31. The seroprevalence between strains B 31 and K 24, B 31 and Ir 105, B 31 and Ir 112, and B 31 and V 123 differed statistically significantly (test χ^2 $p < 0.05$). However, the differences between strains K 24, Ir 112, Ir 105 and V 123 were insignificant. Differences in seroprevalence were insignificant in various regions. Because seroprevalence distribution differs according to a horse's use, no significant differences in seroprevalence were found in all examined horses (Tab. 2).

Consistency of positive and negative findings between American and Slovak strains ranged from 50.5% to 62.4% (Tab. 3 A, B, C, D). When comparing Slovak strains (Ir 105, Ir 112, K 24 and V 123), consistency of positive and negative findings was higher (Tab. 3 E, F, G, H, J). The highest consistency of findings was reached comparing strains Ir 112 and K 24 (*B. garinii* - eastern Slovakia and *B. garinii* - western Slovakia) where consistency of findings reached 95.04%. The same high consistency of findings was observed between the strains Ir 105 and V 123 (*B. burgdorferi* s.s. - eastern Slovakia and *B. burgdorferi* s.s. + *B. afzelii* - also in eastern Slovakia), where consistency of findings reached 89.0%, and also between Ir 112 and Ir 105 with consistency 88.0% (Tab. 3 F, H, I). Higher consistency of findings of serologically examined horses with geographically close strains is in accordance with greater similarity of protein profiles of Slovak strains (Fig. 1, line 2-5) compared to the American strain (Fig. 1, line 1).

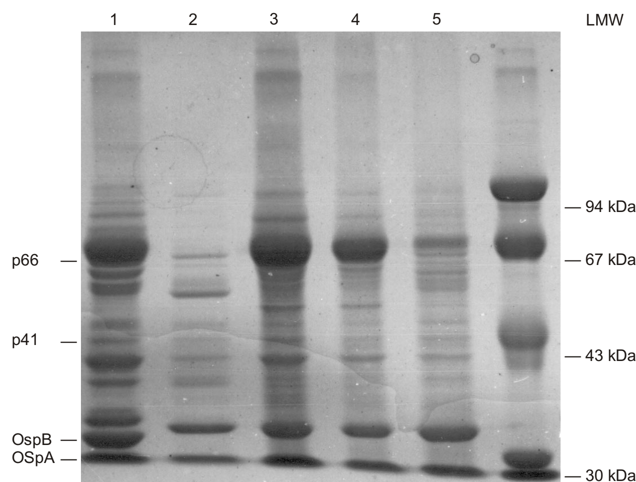

Figure 1. Polyacrylamidogel electrophoresis (SDS-PAGE) of *Borrelia burgdorferi* sensu lato strains: 1- B 31, 2 - K 24, 3 - Ir 105, 4 - Ir 112, 5 - V 123 and low molecular weight standard.

Interassay reproducibility of ELISA. Mean absorbance variation coefficient reached a higher value in positive sera-with each antigen up to 15% than in negative sera - with each antigen up to 10%. A repeated examination of sera with the lapse of 2 months showed 100% reproducibility of the test (positive sera were positive and negative remained negative).

Cross-reactions. Of all the sera from our group of horses positive for borreliosis with particular strains (B 31, Ir 105, V 123, K 24 and Ir 112), 2 sera were also positive for leptospira serovars. One serum reacted to *L. icterohaemorrhagiae* and *L. grippotyphosa* and one to *L. canicola*).

Table 1. Seroprevalence in horses for *B. burgdorferi* by districts.

District	Examined	Seroprevalence (%)				
		B 31*	K 24	Ir112	Ir105	V123
Košice	38	8 (21.05)	20 (52.60)	20 (52.60)	23 (60.50)	22 (57.90)
Prešov	29	8 (27.60)	14 (48.20)	14 (48.20)	15 (51.70)	16 (55.17)
Poprad	18	5 (27.70	7 (38.80)	8 (44.44)	8 (44.44)	6 (33.33)
Stropkov	16	4 (25.00)	6 (37.50)	6 (37.50	7 (43.75)	7 (43.75)
Total	101	25 (24.70)	47 (46.50)	48 (47.50)	53 (52.40	51 (51.49)

* significantly lower compared to K 24, Ir 112, Ir 105 and V 123 for each district

Table 2. Seroprevalence in horses for *B. burgdorferi* by their utilization.

Group of horses	Examined	Seroprevalence (%)				
		B 31	K 24	Ir 112	Ir 105	V 123
Breeding	9	2 (22.22)	3 (33.33)	4 (44.44)	4 (44.44)	5 (55.55)
Race	15	5 (33.33)	8 (53.33)	8 (53.33)	9 (60.00)	8 (53.33)
Draft	77	18 23.40)	36 (46.75)	36 (46.75)	40 (51.94)	38 (49.35)
Total	101	25 (24.70)	47 (46.50)	48 (47.50)	53 (52.40)	51 (51.49)

Table 3. Comparison of anti-*Borrelia* IgG antibodies by ELISA in horses with different whole-cell antigens.

A		B 31		Total
		+	-	
K24	+	15	32	47
	-	1	44	54
Total		25	76	101

Consistent findings (-, +): 58.4%

B		B 31		Total
		+	-	
Ir 105	+	14	39	53
	-	11	37	48
Total		25	76	101

Consistent findings (-, +): 50.5%

C		B 31		Total
		+	-	
Ir 112	+	14	34	48
	-	11	42	53
Total		25	76	101

Consistent findings (-, +): 55.4%

D		B 31		Total
		+	-	
V 123	+	19	32	51
	-	6	44	50
Total		25	76	101

Consistent findings (-, +) : 62.4%

E		K 24		Total
		+	-	
Ir 105	+	38	15	53
	-	9	39	48
Total		47	54	101

Consistent findings (-, +): 76.2%

F		K 24		Total
		+	-	
Ir 112	+	45	3	48
	-	2	51	53
Total		47	54	101

Consistent findings (-, +): 95.04%

G		K 24		Total
		+	-	
V 123	+	37	14	51
	-	10	40	50
Total		47	54	101

Consistent findings (-, +): 76.2%

H		Ir 105		Total
		+	-	
V 123	+	46	5	51
	-	7	43	50
Total		53	48	101

Consistent findings (-, +): 89.0%

I		Ir 112		Total
		+	-	
Ir 105	+	44	9	53
	-	4	44	48
Total		48	53	101

Consistent findings (-, +): 88.0%

J		Ir 112		Total
		+	-	
V 123	+	39	12	51
	-	9	41	50
Total		48	53	101

Consistent findings (-, +): 79.2%

DISCUSSION

Borrelia burgdorferi was described as a single species [20], but later a significant genetic and geographical diversity was confirmed. At least 11 different genospecies have been described [44, 27]. In central Europe the following genospecies have been detected: *B. afzelii*, *B. garinii*, *B. burgdorferi* s.s., *B. valaisiana* and *B. lusitanie* [8, 11, 13]. In western Slovakia, using genotyping analysis, the tick isolates there were identified as *B. garinii* from *I. ricinus* and isolates from reservoir *Apodemus* sp. as *B. afzeli* [23]. Mateička *et al.* [33]. and Gern *et al.* [11] confirmed these genospecies as the most numerous in west Slovakia. Similarly, Hanincová *et al.* [13] report a great representation of *B. garinii* and *B. afzelii* in ticks *I. ricinus*. Apart from the mentioned species, they have also found the following genospecies in western Slovakia, *B. valaisiana* and *B. burgdorferi* s.s. Tresová *et al.* [42]. detected the first isolates *B. burgdorferi* s.l. from *I. ricinus* in eastern Slovakia; later, Štěpanová *et al.* [41] identified *B. burgdorferi* s.s. for first time and other *B. garinii* and *B. afzelii* (Derdáková personal communication) using genotyping analysis confirmed a great representation of *B. burgdorferi* s.s. in eastern Slovakia, and also detected *B. garinii*, *B. afzelii* as well as *B. valaisiana*.

Comparison 5 isolates of *B. burgdorferi* s.l. using ELISA method on group of horses from eastern Slovakia has confirmed the variability which was manifested by different seroprevalence. Slovak strains of *B. burgdorferi* s.s. Ir 105 (52.4%), *B. burgdorferi* s.s. + *B. afzelii* V 123 (51.49%), *B. garinii* K 24 (46.5%), *B. garinii* Ir 112

(47.5%) were more sensitive and showed a higher consistency of findings than the American strain of *B. burgdorferi* s.s. (24.7%).

In our preliminary findings [40] we found a higher sensitivity of local *B. burgdorferi* s.s. and *B. garinii* strains than in the American strain of *B. burgdorferi* s.s. in a group of dogs form the Košice region. We reached the same conclusion as the Swedish authors [5] who, by using ELISA method with a local strain of *B. garinii*, detected a higher number of seropositive patients in an endemic area for Lyme borreliosis than with the American strain. Whereas, the sera of north American patients with LB responded significantly more sensitively with the American strain of *B. burgdorferi* s.s. Norman *et al.* [34] tested the sera of patients with various clinical symptoms from 8 geographical regions and found that European sera responded more significantly with antigens of *B. garinii* and *B. afzelii* in Westernblot, and north American sera with *B. burgdorferi* s.s. Magnarelli *et al.* [29, 30], on the basis of examinations of 8 strains of *B. burgdorferi* s.l. from various sources and regions on a group of different kinds of mammals and patients with clinical and suspect type of borreliosis, did not observe significant differences in sensitivity or distinctiveness, but the employment of local antigens presented a higher level of sensitivity. We and the above stated authors found that differences in sensitivity give the evidence of antigen variability observed among the strains of *B. burgdorferi* s.l. [45].

Even though we used 2 antigens of the same genetic group of *B. burgdorferi* s.s. (B 31 and Ir 105), significant differences in sensitivity could have been influenced by a various host source of antigens (*I. ricinus*, *I. scapularis*) which came from different geographical areas. Antigens of genetically distinct groups of *B. garinii* (Ir 112 and K 24) and *B. burgdorferi* s.s. (Ir 105, V 123) from the same source and area responded more sensitively and findings for them were more consistent, which was proved by protein profiles of Ir 105 and Ir 112 strains which are very similar (Fig. 1, line 3, 4).

In particular districts, the seroprevalence is influenced by the occurrence and different degree of tick infection in territories. Tick *I. ricinus* are infected with *B. burgdorferi* almost all over the territory of Slovakia, with differences in individual localities [22] as well as in individual localities within 1 particular district [36].

With regard to the diagnostic specifity, cross-reactivity with heterologus *Leptospira* antigens was minimal. Serum examinations of horses for *Malleous*, *Brucellosis*, *Equine Coital Exanthema*, *Infectious Anemia* and *Rhinopneumonitis* were negative. According to Luft *et al.* [26], sonicated antigens contain more than 100 proteins, some of which are equivalent to antigens of more than 60 different bacterial species. Some of them may cross-react with borreliae. Hansen *et al.* [14] hold the opinion that serological tests using whole-cells antigens are of low diagnostic specificity. Several strategies for increasing both sensitivity and specificity have been developed, for instance: preabsorbtion of cross-reactive antibodies with *Treponema phagadensis* [48], the use of detergent extracts of *Borrelia burgdorferi* s.l. [4] and the use of purified flagella [15] or various recombinant antigens [39]. In Europe, the extent of variation resulting from the use of different strains for antigenic preparation is still widely discussed [29, 30, 45].

Our results indicated that antigens of Eurocarpathian region reacted in ELISA more sensitively compared with American antigen, and demonstrated potential importance of selecting geographically close isolates as antigens for serological diagnostic of Lyme borreliosis.

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