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ELISA COMPARISON OF THREE WHOLE-CELL ANTIGENS OF *BORRELIA BURGDORFERI* SENSU LATO IN SEROLOGICAL STUDY OF DOGS FROM AREA OF KOŠICE, EASTERN SLOVAKIA

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Abstract: Three antigens of *Borrelia burgdorferi* sensu lato (*B. burgdorferi* sensu stricto - Slovak strain Ir 105, *B. garini* - Slovak strain K 24 and *B. burgdorferi* sensu stricto - American strain B 31) were compared by ELISA on a group of dogs from urban agglomeration of Košice, eastern Slovakia. Of 256 serum samples from dogs examined for the presence of anti-*Borrelia* IgG antibodies, 128 (50%) were positive with Ir 105 antigen, 107 (41.7%) with K 24 and 74 (28.9%) with B 31. The seroprevalence between strains B 31 and K 24 and B 31 and Ir 105 differed statistically significantly (test χ^2, $p < 0.05$), however, the difference between strains K 24 and Ir 105 was insignificant. A significantly higher seroprevalence of all the strains examined was detected in hunting dogs (test χ^2, $p < 0.05$) when compared with service and pet dogs. The seroprevalence correlated with the frequency of outing the dogs in woody areas with the occurrence of borreliae in ticks ($R = 0.5$ or 0.7) as well as with the frequency of finding engorged ticks ($R = 0.5$). An epizootiological anamnesis showed a fair specificity in all the strains examined. The Slovak strains showed the higher consistency of positive and negative findings (70%) but statistically lower specificity than the American strain.

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Key words: *Borrelia burgdorferi* sensu stricto, *Borrelia garini*, ticks, dogs, seroprevalence, ELISA, sensitivity, specificity, epidemiology, borreliosis, West Carpathians, Slovak Republic.

INTRODUCTION

Lyme borreliosis is a zoonotic disease, evoked by the spirochete *Borrelia burgdorferi*. Besides man, various free-living and domestic animal species are exposed also worldwide to this zoonosis. From the epidemiological point of view, dogs are very important since they are considered a suitable indicator of the spread of human borreliosis [10].

The diagnosis of Lyme borreliosis is based on the epidemiological and epizootiological case history, clinical symptoms, laboratory tests and prompt antibiotic response [3, 6, 30]. In view of the frequent asymptomatic course of the disease (particularly in animals) and its non-specific clinical symptoms, the diagnosis of Lyme borreliosis is based on laboratory techniques. Polymerase chain reaction (PCR) for the evidence of agent's DNA in tissues or body cavities and electron microscopy are highly sensitive and specific techniques [9, 13, 34, 36], which have been used only experimentally. Methods for the assessment of antibody function using complement fixation [21, 22], and growth inhibition of borreliae [38,

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44] are also very limited. Serological tests based on the bond of antibodies and antigens, fixed on the solid phase, are the most frequently employed tests in the current routine diagnosis of the disease. They include indirect flourescent-antibody assay (IFA), indirect enzyme-linked immunosorbent assay (ELISA) [5, 14, 27, 42] and Western immunoblot [8, 24]. ELISA is most frequently used because of its more simple technical equipment and result interpretation. In the past decade, numerous commercial sets have been developed in the USA, Europe and Asia, primarily for use in human medicine [23, 30, 32]. Strain B 31 from Shelter Island, New York, is often used as antigen for diagnosis. In addition to this strain, antigenic properties of other strains of *B. burgdorferi* sensu lato have also been studied in order to determine whether local strains are indispensable for optimal serological diagnosis [30, 31].

The objective of this study was to compare three *Borrelia* strains as antigens by performing ELISA in a group of dogs from eastern Slovakia. Two of those strains were isolated from the ticks *Ixodes ricinus* on the territory of Slovakia and one from American ticks *I. scapularis*.

MATERIALS AND METHODS

Sera. The dogs came from the urban area of Košice, situated on the interface between a submontane wooded and a lowland agricultural landscape (180–350 m above sea level) in the southeastern part of the West Carpathians. The northern peripheral part of the area is composed of a hornbeam-oak park forest with a frequent occurrence of ticks *I. ricinus*. The tree-verdure in its central part is made up of several small parks with a low density of ticks.

During the years 1993–1996, serum samples were taken from 256 clinically healthy dogs of different race and age and of either sex. The sera were provided by the dog owners responding to an appeal for examination of dogs for the presence of anti-*Borrelia* IgG antibodies at police units (60), hunting organizations (75) and veterinary outdoor departments (121). The epizootiological anamnesis was taken with reference to the race, age, sex, frequency of outing the dogs in tick-infested wooded areas and frequency of finding engorged ticks during the vegetation period.

Antigens. The following whole cells sonicated cultures from *B. burgdorferi* sensu lato were used as antigens: *B. burgdorferi* sensu stricto Ir 105 - our own local strain isolated from *I. ricinus* from the urban area of Košice, eastern Slovakia [47], *B. garini* K 24 - isolated from *I. ricinus* from western Slovakia [33] and *B. burgdorferi* sensu stricto B 31 - American strain isolated from *I. scapularis* [18]. Antigens from the strains B 31 and K 24 were obtained by generosity of the Institute for Research of Rheumatic Diseases in Piešťany.

Antigen from Ir 105 strain was produced as follows: Borreliae were incubated at 33°C in BSK - II medium (Sigma) at 7-day intervals. The culture was centrifuged at 10 000 g for 30 min, washed three times in PBS (pH 7.2, with 5 mM $MgCl_2$) and sonicated at 20 KHz for 3 min with cooling at 20–30 W (Sonic Dysmembrator, Dynatech, UK). The culture was then again centrifuged at 10 000 g for 30 min. The supernatant was used as antigen. 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.

ELISA. Sera were examined by a modified ELISA, available in commercial sets (Institute for Sera and Vaccines, Prague) used for diagnosis of Lyme borreliosis in human medicine, in the following way: Microplates were parallelly filled 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) and incubated overnight at 4°C. After washing three times with phosphate buffer (pH 7.2) containing 0.05% Tween 20, 100 µl portions of sera diluted at 1:400 in phosphate buffer with 0.05% Tween 20 and 1% BSA, and *Treponema pallidum* standard lyophilized extract (FTA sorbent) were added to each well and incubated at 37°C for 30 min. After a triple washing of the plates, 100 µl portions of anti-dog IgG peroxidase conjugate (Sigma) were added per well, diluted at 1:2000 for B 31, and 1:3000 for K 24 and Ir 105. After 30 min of incubation and a subsequent washing, 100 µl per well of substrate solution (0.1 M citrate buffer pH 5.0 with 0.02% H_2O_2) with orthophenylene diamine were added. The reaction was stopped with 5% H_2SO_4 after 15 min of incubation. The absorbance was measured at 492 nm. The serum from a dog with clinical manifestations of Lyme borreliosis was used as a positive control. Mean absorbance values of the above serum in ELISA (for B 31 = 1.35, for K 24 = 1.48 and for Ir 105 = 1.54) were calculated from the absorbance mean obtained by repeated titrations and used as standard positive controls. As negative controls were used the dog sera negative to antigens tested in repeated titrations ($E_{492} < 0.4$), which were collected from an area where ticks are of the rare occurrence. A coefficient *k* was calculated for each microplate as the proportion of mean absorbance value in standard positive control to the absorbance value in positive control measured in the test. Mean absorbance values of all sera were multiplied by this coefficient. Sera with the calculated absorbance value $E_{492} > 0.6$ were considered positive.

Interassay reproducibility of ELISA: Parallelly with each strain, 10 sera were repeatedly (10 times) examined over 6 months, of which 3 were strongly positive ($E_{492} > 1.2$), 3 medium positive ($E_{492} = 0.7 - 0.9$) and 4 negative ($E_{492} < 0.4$). All the sera were repeatedly examined with the lapse of 6 months with antigens of strains Ir 105 and K 24 and with the lapse of 1–3 years with antigen of strain B 31.

Statistical evaluation was done by test χ^2, by Spearman's rank correlation coefficient and by coefficient of variation (V%) [41]. Sensitivity and specificity were calculated after Mittenecker [35].

Table 1. Seroprevalence of *B. burgdorferi* in various dog groups.

Groups of dogs	No. of examined dogs	No. of positive (%)		
		B 31*	K 24	Ir 105
Hunting†	75	34 (45.3)	46 (61.3)	53 (70.6)
Private guard dogs	53	17 (32.1)	25 (47.2)	30 (56.6)
Service	60	11 (18.3)	18 (30.0)	21 (35.0)
Pet	68	12 (17.6)	18 (26.5)	24 (35.3)
Total	256	74 (28.9)	107 (41.7)	128 (50.0)

†significantly higher compared to service and pet dogs for each of B 31, K 24, Ir 105 antigens ($p < 0.05$, test χ^2); *significantly lower compared to K 24 and Ir 105 antigens for each group of dogs ($p < 0.05$, test χ^2).

Table 3. Seroprevalence of *B. burgdorferi* in dogs according to exposure frequency of engorged ticks.

Frequency of ticks	No. of examined dogs	No. of positive (%)		
		B 31	K 24	Ir 105
Often	62	24(38.7)	37 (59.6)	44 (70.1)
Sporadically	92	22 (23.9)	34 (37.0)	41 (44.6)
Not given	102	28 (27.4)	36 (35.3)	43 (42.1)
Total	256	74 (28.9)	107 (41.7)	128 (50.0)
Spearman coefficient of correlation:		R = 0.5	R = 0.5	R = 0.5
Probability value:		$p < 0.01$	$p < 0.01$	$p < 0.01$

RESULTS

Of 256 sera of dogs from the region of Košice (Tab. 1) examined for the presence of anti-*Borrelia* IgG antibodies 128 (50%) were found to be positive using antigen Ir 105, 107 (41.7%) with antigen K 24 and 74 (28.9%) with antigen B 31. The seroprevalence between strains B 31 and K 24 and between B 31 and Ir 105 varied statistically significantly (test χ^2, $p < 0.05$), however, the comparison between strains K 24 and Ir 105 showed insignificant differences. All the strains examined showed a significantly higher seroprevalence in hunting dogs (test χ^2, $p < 0.05$) compared with service and pet dogs. The values for seroprevalence showed its dependence on the frequency of outing dogs in woody areas with the presence of *Borrelia*-infected ticks with all the antigens examined (R = 0.5 or 0.7, Tab. 2). A similar dependence was also observed as regards the frequency of finding the engorged ticks recorded in the anamnesis (R = 0.5, Tab. 3).

Sensitivity calculated from consistency of positive findings between particular antigens was lower for B 31 (44% and 43%, respectively) than that for K 24 and Ir 105 (63.5% and 74.3%, respectively). On the contrary, specificity calculated from consistency of negative findings for B 31 reached 81.8% and 85.1%, respectively, while that for K 24 and Ir 105 was lower, 67% and 60%, respectively. K 24 compared with Ir 105 showed lower differences in sensitivity (61.7% and 73.8%, respectively) and specificity (78.1% and 67.1%). Consistency of positive and negative findings between particular antigens were reached in 64–70% (Tab. 4, A, B, C).

Interassay reproducibility of ELISA**:** Mean absorbance variation coefficient reached higher values in highly positive sera (with antigen B 31: 18.8–20.2%, with K 24: 15.8–24.7% and with Ir 105: 17.6–21.3%) than in less positive sera (15.5–17.9%, 10.6–14.9% and 11.3–15.4%, respectively) and negative sera (10.2–12.3%, 13.4–15.1% and 13.8–15.4%, respectively). A repeated examination of sera with the lapse of 6 months or 1–3 years showed a 100% reproducibility of the test (positive sera were positive and negative remained negative).

Table 2. Seroprevalence of *B. burgdorferi* in dogs according to their exposure frequency in tick-infested wooded areas.

Exposure frequency	No. of examined dogs	No. of positive (%)		
		B 31	K 24	Ir 105
Once a week	88	35 (39.7)	49 (55.7)	59 (67.0)
Once a month	45	14 (31.1)	21 (46.6)	25 (55.5)
Once in summer	49	12 (24.5)	15 (30.6)	19 (38.7)
None	47	6 (12.8)	10 (21.3)	12 (25.5)
Not given	27	7 (25.9)	12 (44.4)	13 (48.1)
Total	256	74 (28.9)	107 (41.7)	128 (50.0)
Spearman coefficient of correlation:		R = 0.5	R = 0.5	R = 0.7
Probability value:		$p < 0.01$	$p < 0.01$	$p < 0.01$

DISCUSSION

An ELISA comparison of three strains of *B. burgdorferi* sensu lato on a group of 256 clinically healthy dogs from the region of Košice showed their variable seroprevalence. Slovak strains *B. burgdorferi* sensu stricto Ir 105 (50%) and *B. garini* K 24 (41.7%) were more sensitive and showed a higher consistency of findings than American strain *B. burgdorferi* sensu stricto B 31 (28.9%). Isogai *et al*. [17] examined by ELISA sera of 216 healthy dogs using Japanese strains (HO 14 - isolated from *I. ovatus* and HP 3 from *I. persulcatus*) and detected a lower seroprevalence with HP 3 (11.5%) than with HO 14 (14.3%). Magnarelli *et al*. [30] compared by ELISA eight strains isolated from different sources and areas (from *Peromyscus leucopus, I. scapularis, I. pacificus* and from human skin from the USA, from *I. scapularis* from Canada and from *I. persulcatus* from Japan and Khabarovsk). A group of positive patients with clinical and suspect diagnosis showed an 83.8–100% sensitivity of IgM antibodies with all the strains. The lowest sensitivity was observed with an American strain *B. burgdorferi* sensu stricto and the highest with Japanese strain *B. garini* and American strains isolated from

Table 4. Comparison of anti-*Borrelia* IgG antibodies by ELISA in dogs with three whole-cell antigens.

A.

		B 31		Total
		+	-	
K24	+	47	60	107
	-	27	122	149
Total		74	182	256

Sensitivity K 24: 63.5%; Specificity K 24: 67.0%; Sensitivity B 31: 44.0%; Specificity B 31: 81.8%; Consistent findings (- , +) 66.0%.

B.

		B 31		Total
		+	-	
Ir 105	+	55	73	128
	-	19	109	128
Total		74	182	256

Sensitivity Ir 105: 74.3%; Specificity Ir 105: 60.0%; Sensitivity B 31: 43.0%; Specificity B 31: 85.1%; Consistent findings (-, +): 64.0%.

C.

		K 24		Total
		+	-	
Ir 105	+	79	49	128
	-	28	100	128
Total		107	149	256

Sensitivity Ir 105: 73.8%; Specificity Ir 105: 67.1%; Sensitivity K 24: 61.7%; Specificity K 24: 78.1%; Consistent findings (-, +): 70.0%.

P. leucopus and *I. pacificus.* On the other hand, the lowest level of IgG antibodies was detected with American isolate from *I. pacificus* (82.6%), while with other strains the sensitivity exceeded 91%. Magnarelli *et al.* [31] tested the same strains on a group of positive mammals of various species. All those strains showed sensitivity > 80%, except for the strain isolated from *I. persulcatus* from Khabarovsk with sensitivity of only 66%. The differences in sensitivity detected in our study and also by the mentioned authors may by caused by the antigenic variability that exists among strains of *B. burgdorferi* sensu lato [1, 2, 4, 11, 37, 48]. Although strains of *B. burgdorferi* sensu lato seem to have numerous common antigens, mammals may not immunologically recognize a multitude of common epitopes in the same way [31].

Although we worked with two antigens of the same genetic group of *B. burgdorferi* sensu stricto, the significant differences in their sensitivity could have been caused by the different host source of the antigens (*I. ricinus*, *I. scapularis*) that came from different geographic territories. The antigens of genetically different groups of *B. garini* and *B. burgdorferi* sensu stricto from the same biological source and the same geographic territory (the West Carpathians) responded more sensitively and their findings were more consistent. It is common knowledge that besides its natural heterogeneity, *B. burgdorferi* is subject to genotype and phenotype changes during incubation *in vitro*. The loss of its virulence is associated with the loss of plasmids, reflected in the changed content of proteins [43]. The higher sensitivity of Slovak strains and their strikingly varied responsiveness when compared with American strain could have been also a consequence of a different number of passages (Ir 105 4 passages, K 24 > 5 passages, conversely B 31 > 30 passages).

Ticks *I. ricinus* are infected with *B. burgdorferi* sensu lato almost all over the territory of Slovakia, showing a notable local and seasonal variability with individual regions [18]. Reports on the epidemiological situation in Slovakia (prepared by the Specialised State Medical Institute in Banská Bystrica) suggest that the number of human patients with borreliosis in 1988–1996 ranged between 59–998 cases, peaking in 1993. A similar course of the incidence was also recorded in eastern Slovakia with the highest seroprevalence of anti-*Borrelia* IgG antibodies detected in dogs (27%) with American strain of *B. burgdorferi* sensu stricto B 31 [45]. *B. burgdorferi* infection of ticks in 1991–1995 in the suburban park forests of Košice reached 2.1–41.7% with its highest rate (16–41.7%) observed in the localities frequented also by most of our examined dogs [39, 40]. This rate of infection seems to have been reflected in a higher seroprevalence detected in dogs by using Slovak strains.

Our results also prove that from the epidemiological aspect the dog is a risk animal since it is a constant companion of man in places where he comes into contact with infected ticks. The removal of ticks and handling the animal after returning from outdoors is also dangerous. The frequent findings of ticks in hunting dogs and their high seroprevalence also intensify the risk. In addition to the tick *I. ricinus*, also *I. hexagonus* can play a role as a vector of the agent of Lyme borreliosis [12]. The latter is a common parasite of carnivores maintained in kennels, which increases the possibility of human infection through guard dogs. A more detailed analysis of the seroprevalence in terms of the individual groups of dogs, their age, sex, frequency of finding the engorged ticks and frequency of outing dogs in wooded areas with *Borrelia*-infected ticks has already been discussed [45, 46].

As regards the diagnostic specificity, epizootiological anamnesis of all dogs examined has shown a direct dependence of seroprevalence on the frequency of outing the dogs in wooded areas with the presence of infected ticks and on the frequency of finding engorged ticks. Statistically, Slovak strains showed a lower specificity than the American strain. Considering the fact that sonicated antigens contain more than 100 proteins [26], some of which are equivalent to antigens of more than 60 different bacterial species, it is very difficult to determine exactly the specificity if clinical symptomatology is

unavailable. According to Hansen *et al.* [16], serological tests using whole-cell antigens are of a low diagnostic specificity. Magnarelli *et al.* [30, 31], however, have conducted comparative studies with whole-cell antigens proving their high sensitivity and specificity. According to these authors, an optimal serological diagnosis does not necessarily require the use of local strains. Local strains are preferred for their availability; however, if the antigenic structure varies considerably among *B. burgdorferi* sensu lato strains in various geographic territories. In such cases, the selection of individual strains should be taken into account. In terms of the variable host immune response to various antigens (weak response at early stage), search is going on for mixtures of highly specified and purified antigenic subunits, namely recombinant proteins, flagellar and outer surface proteins A and B, which increase the sensitivity and specificity of tests [7, 15, 19, 28, 29].

CONCLUSION

Preliminary results indicate that the Slovak strains of *Borrelia burgdorferi* sensu lato Ir 105 and K 24 are more sensitive in serological testing for borreliosis and show a higher consistency of positive and negative findings than the American strain B 31. To verify the applicability of our own Slovak strain Ir 105 as an antigen for use in serological diagnosis of Lyme borreliosis in Slovakia, it is necessary to continue with its testing by ELISA on groups of animals from different areas, or on human patients with clinically confirmed borreliosis and by employing Western blot immunoanalysis for a more precise evaluation.

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