ORIGINAL ARTICLES AAEM

Ann Agric Environ Med 2008, 15, 85–90

SEROPREVALENCE OF ANTI-*BORRELIA BURGDORFERI* ANTIBODIES IN DOGS AND HORSES IN TURKEY

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Bhide M, Yilmaz Z, Golcu E, Torun S, Mikula I sr.: Seroprevalence of anti-*Borrelia burgdorferi* antibodies in dogs and horses in Turkey. *Ann Agric Environ Med* 2008, **15**, 85–90.

Abstract: The aim of the study was to determine the seroprevalence of anti-*Borrelia burgdorferi* antibodies in a population of Turkish dogs and horses, as well as to compare the sensitivity of novel flow-cytometry-based borreliacidal antibody test (BAT) with ELISA assay. Serum samples collected from 400 dogs and 300 horses were tested with enzyme-linked protein A/G assay (ELPAGA), using *Borrelia* whole cell antigens. ELPAGA test showed 93 dogs (23.2%) and 18 horses (6%) serologically positive for anti-*Borrelia* antibodies. In parallel testing of sera with BAT, we found 27.75% positive dogs and 6.33% positive horses. When the results of these serological testes were compared with the health status of the animals, the most common clinical signs noticed in dogs were skin manifestations, urinary tract disorder and anemia; however, no clinical symptoms were observed in horses positive for the anti-*Borrelia* antibodies. This is a first time that seroprevalence of Lyme disease in dogs and horses has been reported from Turkey, as well as the use of novel BAT in animals.

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Key words: ***Borrelia burgdorferi*, Lyme disease, ELPAGA, Borreliacidal antibody test, dog, horse.**

INTRODUCTION

Lyme borreliosis (Lyme disease) is a multisystemic zoonotic disease caused by the *Borrelia burgdorferi* sensu lato complex, usually transmitted by ticks of the genus *Ixodes* in Europe [1, 2, 21, 23, 25, 30]. A variety of small mammals and birds are of great importance as reservoirs for *B. burgdorferi* [4, 20, 25]. Many different *Borrelia* spp. have been isolated from reservoir animals, tick vectors and Lyme disease patients in different parts of the world, including Turkey [17, 18, 22, 28, 30]. *B. burgdorferi* was at first thought to be homogenous, but is now classified into separate genospecies such as *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* [22].

B. burgdorferi may causes recognizable clinical signs of disease in dogs, horses, cattle, and sheep [1, 13, 25]. Lyme disease in humans is characterized by influenza-like symptoms, mostly associated with erythema migrans, and occasionally followed by cardiac, neurological, or arthritic form [9, 15]. However, not all infected dog and horses develop clinical signs of Lyme borreliosis [15]. Clinical signs can be observed in approximately 5–10% of animals exposed to *B. burgdorferi* [1, 26]. Clinical illness in dogs presents as an acute onset of single or shifting limb lameness, swollen joints, fever and depressed attitude [13]. Less commonly observed clinical signs include behavioural changes, seizures, encephalitis, renal dysfunction, cardiac arrhythmia, and reproductive disorders [1, 15]. Unlike in humans, erythema migrans is reported rarely in dogs. In horse abortion, laminitis and weight loss have been identified as a sequelae to *B. burgdorferi* sensu lato infection [15, 19, 29]. Moreover, in horses, neurological signs (head tilt,

Received: 1 May 2007
Accepted: 30 March 2008

difficulty in swallowing, aimless wandering) and blindless have been reported as a consequence of Lyme disease associated with encephalitis or panuveitis [14].

Lyme disease in animals can be diagnosed based on a combination of the following criteria: 1) presence of typical clinical symptoms; 2) exclusion or differential diagnoses; 3) explicit response to antibiotic; 4) evidence of tick contact in an endemic area; and/or 5) presence of antibodies in serum which can be accepted as a major diagnostic indicator. Due to the lack of pathognomonic clinical signals in Lyme disease, serology has become a helpful and widely used tool in diagnostics and epidemiological surveys [1, 3, 21, 30]. To test *B. burgdorferi* infection, different serodiagnostic techniques such as indirect fluorescent antibody test (IFA), enzyme linked immunosorbent assay (ELISA), enzyme linked protein A or G assay (ELPAGA), coupled with confirmatory western blotting are used [3, 23, 27, 31, 32]. Detection of *B. burgdorferi* by dark-field microscopy, immunostaining, culture in Barbour-Stoenner-Kelly (BSK) medium, and polymerase chain reaction (PCR) analysis of body fluids and tissues has been reported in both naturally and experimentally infected dogs [1, 21, 29]. However, till to date, few studies are available that report borreliacidal antibody test (BAT). One of the advantages of BAT is its use in detection of antibodies in wild and free living animals where species specific conjugates in ELISA based assays are not easily available.

Borelia seroprevalence has been recorded in humans in some rural areas of Turkey (24); hitherto no data has been available for dogs and horses. Therefore, the aim of the study was to investigate the seroprevalence to anti-*Borrelia burgdorferi* antibodies in dogs examined at an animal hospital and actively racing horses in Turkey, with ELPAGA and novel BAT techniques.

MATERIALS AND METHODS

Animals

Dogs. Routine physical and laboratory examinations were performed for the diagnostic work-up related with the clinical signs or general health status in 400 dogs admitted to the small animal clinic of internal medicine of the veterinary faculty, University of Uludag (Bursa-Turkey). Physical examinations included body temperature, heart and respiratory rates, external lymph nods palpations, mucous membranes, lung and heart auscultations, etc. In the case of laboratory assessement, blood samples were collected into the vacuteiner tubes without anticoagulant (EDTA) (Becton Dickinson, 5–10 ml, Belgium). Serum was separated for serological assays. None of the dogs had been vaccinated against Lyme disease.

Horses. Blood samples were collected in the Clinic of the Jockey Club in Izmir, Turkey, during a routine check-up of 300 horses which appeared to be healthy and actively racing. All horses were vaccinated at least once with Prevac T Pro vaccine (inactivated vaccine against equine influenza and tetanus, Intervet, UK).

Serum samples collected from the dogs and horses were stored at -20ºC untill use.

Enzyme linked protein A/G assay (ELPAGA)

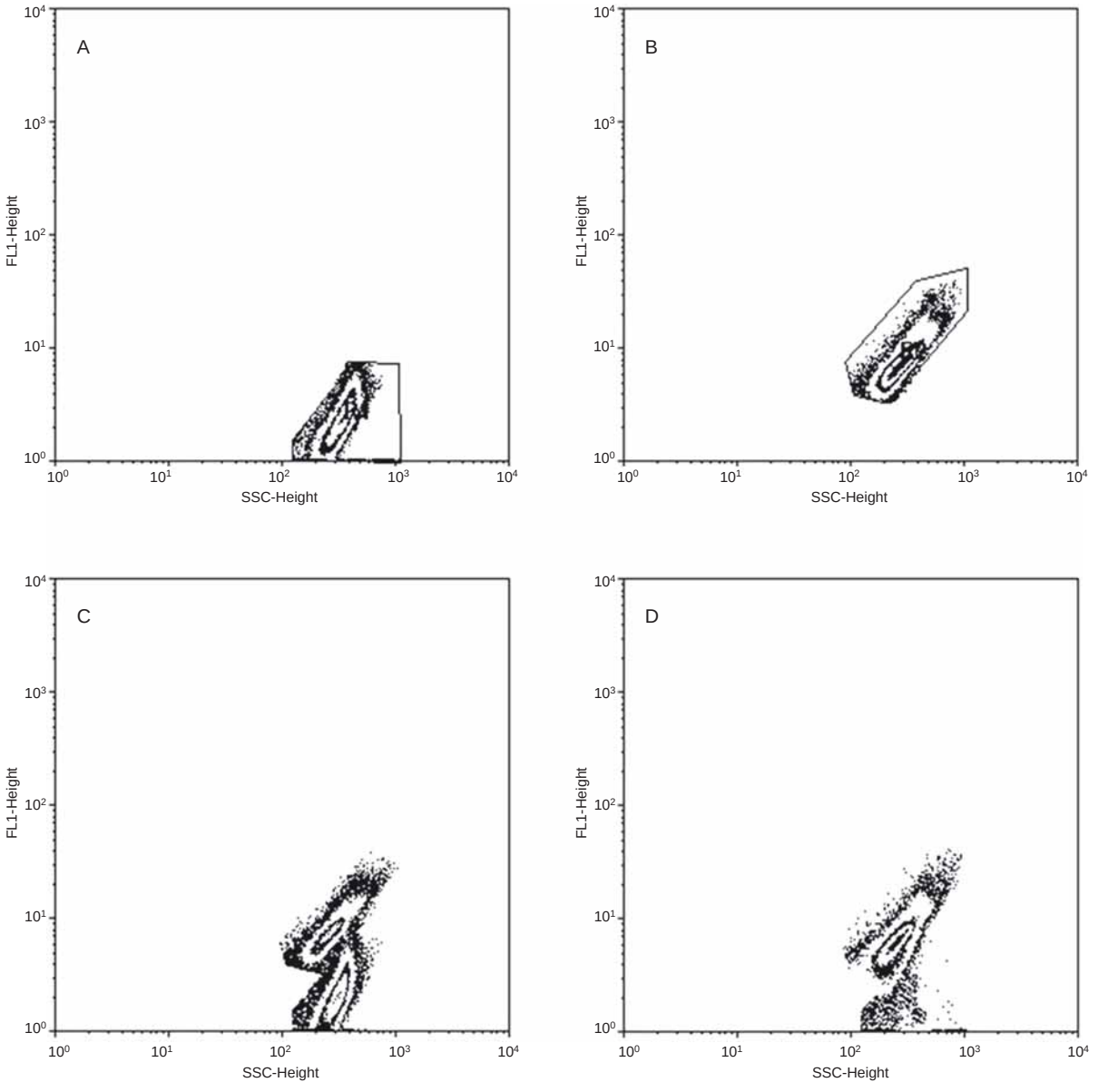
ELPAGA was performed as described earlier by Bhide *et al.* [3]. In short, a mixture of *Borrelia* whole cell antigens (*B. garinii*, SKT-3 serotype 6, *B. afzelii*, PKo serotype 2 and *Borrelia burgdorferi* sensu stricto, SKT2, serotype 1) were used to coat microtiter wells (10 µg/ml). The mixture of this whole cell antigens had good affinity towards antibodies against all three major *Borrelia* genospecies found in Europe: *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* [3]. Non-specific binding sites were blocked by the addition of 100 µl/well of 1% bovine serum albumin (Sigma). Diluted (1:300) canine and equine serum samples were added in duplicate (100 µl/well) to the wells. Positive and negative control sera were also diluted accordingly. After incubation for 1 h at 37ºC, plates were washed three times with phosphate buffer saline plus Tween20 ($PBST_{20}$). Protein A/G conjugated with horse radish peroxidase (HRPO) was diluted at 1:2700 in $PBST_{20}$ and added (100 µl/well) to each well. Plates were incubated for 1 h at 37ºC and washed three times with $PBST_{20}$. ABTS substrate (2,2'Azino-di-3-ethyl-benzothiazolin-sulfonate; Sigma) was added (100 µl/well) and the plates incubated for 25 min. Absorbance was measured at 405 nm. Cut-off value for dog and horse was kept as 1.8 absorbance.

Borreliacidal antibody test (BAT)

Preparation serum samples and *Borrelia* strain for BAT. Serum samples were filtered through 0.22 µm filter (Minisoft) and incubated at 56ºC for 10 min to inactivate complement. History of patients was checked for recent antibiotic treatment which can give false positive results. Positive control serum was diluted serially to standardize the borreliacidal effect on percent shift in fluorescence intensity, as well as to assess the sensitivity of BAT assay. All test sera were diluted 1:5 in sterile BSK II medium.

Borrelia strain SKT-2 (*B. burgdorferi* s.s.) was grown to mid-log phase (approximately 72 hours) at 35ºC. This strain was selected because of its relatively faster growth and the excellent peculiar mobility of spirochetes in normal culture. Concentration of borrelial cells was adjusted up to 10^4 cells/ml. Dead control of *Borrelia* was prepared by heating at 56ºC for 25 min.

Borreliacidal antibody test (BAT). BAT was performed according to Callister *et al.*, [8]. The amount of *Borrelia* suspension, serum concentration and units of rabbit complement were standardized by checkerboard method. For actual BAT, 50 µl of diluted (in BSK II) and heat inactivated



A – live *Borrelia* (negative) control with gate R1. Note that cell signal is from 10^2–10^3 at SSC height axis, but at FL1 axis, the cell signals are at base level.
B – dead *Borrelia* (positive) control with gate R2. Note that at FL 1 axis cell signals are shifted due to fluorescence of acridine orange entered in to dead borrelial cells.
C – Actual BAT. Serum sample with moderate anti-*Borrelia* antibody titer. Note that cell signals are divided into predictive R1 and R2 gates, indicating partial borreliacidal effect.
D – Actual BAT. Serum sample with high anti-*Borrelia* antibody titer. Few cell signals are noticed in predictive R1 gate, indicating maximum borreliacidal effect.

Figure 1. Detection of borreliacidal antibodies by flow cytometry (BAT).

serum sample was mixed with 50 µl of *Borrelia* suspension. Ten micro liters of sterile rabbit complement (~ 210 CH_{50} units) were also added. The assay plate was gently swirled, sealed with a special cover, and incubated at 32ºC for 14–16 hrs. For growth control (live control), only BSK II medium was added along with *Borrelia* suspension. As a negative control, borreliae were incubated in known negative serum with rabbit complement.

After incubation, borrelial immobility, growth and bleb formation, judged by dark field microscopy, were considered to confirm the borreliacidal activity while standardizing the method. For actual BAT, samples were screened by flow cytometry. In short, 10 µl of incubated suspension was diluted 1:5 with PBS (0.01 Mol, pH 7.2; filtered through 0.2 µm filter) containing 1 µg of acridine orange/ml and incubated for 5 min at room temp. Analysis was carried out on a FACScan flow cytometer (Bacton-Dickinson). For each sample, events were acquired in the list mode for 2 min. The sample fluid flow rate was kept low to reduce signal variability. Controls were also analyzed simultaneously. Data was analyzed by WinMDI software (Version 2.8). *Borrelia* spirochetes were differentiated from BSK and complement particles using fluorescence intensity and side scatter parameters. Non-rectangular gates on the side-scatter vs FL-1 fluorescence dot plot were used to identify spirochetes. Logarithmically amplified fluorescence signals

were used. More than 15% increase in fluorescence intensity compared to that of negative control serum was considered positive. Analyzed data was statistically correlated with live (negative) and dead (positive) controls.

Statistical analysis. For ELPAGA, mean absorbance of each test sample was compared with the earlier determined cutoff value for confirmation of positivity or negativity. The cut off value was set on the basis of the known positive and negative controls. Paired t-test (STATGRAPHICS plus 5.1) was used to assess the age and sex dependent variations, as well as to assess the variation in seroprevalence between healthy and sick dogs. Also, to determine a relationship between seropositivity and clinical problems, chi-square test or Fisher exact test was used. To assess the correlation between ELPAGA and BAT, the kappa test (*k*) was used.

RESULTS

Anti-*Borrelia* antibodies were found in 93 (23.2%) of the 400 dogs tested by ELPAGA, whereas positive samples found by BAT were 27.75% and 6.33% respectively in dogs and horses. Although we found a higher percentage of seropositive animals by BAT, there was no significant variation between ELPAGA and BAT results. The correlation between ELPAGA and BAT was also confirmed by the kappa test ($K = 0.95$). In other words, it indicates that specificity and sensitivity of BAT is equivalent to ELISA. To confirm the sensitivity, in a 10-fold serially diluted known positive serum sample, we detected anti-*Borrelia* antibodies in 10^{-7} and 10^{-9} dilutions by ELPAGA and BAT, respectively. Representative results of BAT are presented in Figure 1.

Amongst seropositive dogs, 34.4% were healthy and 65.6% showed various clinical symptoms (Tabs. 1, 2). Antibody titers in seropositive healthy or sick dogs (Tab. 2) were not significantly different ($p>0.05$) (mean ± SEM; 2.60 ± 0.1 vs. 2.62 ± 0.02). On the basis of the routine physical examinations and laboratory test results (data not shown), we observed that the major clinical problems were skin manifestations, diarrhea and/or vomiting, urinary tract infection and anaemia; heart failure and lameness were less common in seropositive dogs. There was a significant distribution, between seropositivity and clinical problem but only for skin manifestations (DF=1, chi-square = 8.5, $p < 0.01$).

In 93 seropositive dogs, the predominantly affected breeds were mixed or cross-bred dogs (n=26), followed by German shepherd (n=13), Anatolian sheep dog (n=11) and Terrier (n=10) (Tab. 3). The number of male dogs infected (n=55) was higher than female dogs (n=38). However, no significant variation ($p > 0.05$) between male and female seropositivity was observed. As can be seen in Table 4, there was no statistically significant association between age and seroprevalence, but the commonly affected age group in dogs (both healthy or with clinical symptom) was from 7–12 months.

Table 1. Major clinical problems found in seropositive dogs.

Clinical symptom	Number of dogs	Percent of dogs
Skin manifestation	16	26.2
Diarrhoea/vomiting or both	13	21.3
Urinary tract disorder	11	18.0
Anaemia	9	14.7
Coughing	4	6.55
Pyometra	3	4.91
Heart failure	1	1.63
Lameness	1	1.63
Seizures	1	1.63
Reproductive disorder/Abortion	1	1.63
Neoplasia	1	1.63
Total	61	

Table 2. Antibody titers in seropositive healthy or sick dogs.

Dogs	Optical density (OD)			
	1.8–2.0	2.01– 3.0	3.01–3.5	Total
Clinical Symptoms	17	26	18	61
Healthy	7	14	11	32
Total	24	40	29	93

Table 3. Positive and negative test results for anti-*Borrelia* antibodies in dogs of different breed.

Breed	Seronegative	Seropositive	Total
Anatolian sheep dog	44	11	55
Boxer	9	6	15
Cocker Spaniel	8	4	12
G. Retriever	17	1	18
G. Shepherd	32	13	45
Husky	9	3	12
L. Retriever	8	3	11
Mix	74	26	100
Pointer	14	3	17
Rottweiler	16	2	18
Setter	7	5	12
Terrier	33	10	43
Miscellaneous	36	6	42
Total	307	93	400

Horses were of Arabian breed, both sexes (187 female and 113 male) and aged from 3–11 years. 18 positive sera were found: 4 sera showed low antibody titer (1.8–2.0 OD), 4 samples showed intermediate (2.1–3.0 OD) and 10 samples had higher antibody titer (3.0–3.5 OD). The highest incidence was found in the 6–10 year age group. In all of the 10 horses showing high antibody titer by ELPAGA, we found antibody mediated high borreliacidal effect in

Table 4. Prevalence of anti *B. burgdorferi* antibodies among dogs of different age groups.

Age (months)	No. (%) of dogs					
	Healthy dogs			Sick dogs		
	Total	Seropositive	Seronegative	Total	Seropositive	Seronegative
1-6	90 (58)	20 (22.0)	70 (78.0)	69 (28.0)	21 (30.4)	48 (69.6)
7-12	20 (13)	6 (30.0)	14 (70.0)	24 (9.7)	11 (45.8)	13 (54.2)
13-24	11 (7)	1 (9.0)	10 (91.0)	28 (12.1)	8 (28.6)	20 (71.4)
25-48	24 (15)	2 (8.0)	22 (92.0)	35 (14.2)	6 (17.2)	29 (82.8)
≥49	11 (7)	3 (27.0)	8 (73.0)	88 (35.7)	15 (17.0)	73 (82.9)
Total	156	32 (20.5)	124 (79.5)	244	61 (25.0)	183 (75.0)

BAT, ranging from 86–95%. On the other hand, we found 35–66% borreliacidal effect in 8 samples which showed either low or intermediate antibody titer in ELPAGA. One sample which was negative in ELPAGA showed 38.6% borreliacidal effect in BAT, and was thus considered as positive for the presence of anti-*Borrelia* antibodies.

DISCUSSION

Infection with *B. burgdorferi* induces the production of borreliacidal antibodies [5] that activate complement to form a membrane attack complex. Callister *et al.*, [7] have successfully shown the borreliacidal activity of Lyme disease antibodies, and that the percentage of killing was correlated with the duration and course of disease. The use of BAT in animals, particularly in dogs, was successfully undertaken with no conciliation in sensitivity and specificity [8]. Moreover, the authors have reported the detection of anti-*Borrelial* antibodies in 73% dogs 1 week after infection and in 100% after 3 weeks of infection. Though the sensitivity and specificity of BAT is proved to be higher, the requirement of flow cytometer hinders its common use in serodiagnosis.

Lyme disease has been reported in many countries of Europe, America and Asia; however, knowledge about its epidemiology in Turkey is deficient. We have reported here, for the first time, the presence of *B. burgdorferi* antibodies in dogs and horses in Turkey. Considerable seroprevalence indicates the exposure of dog and horse populations to *B. burgdorferi* in Bursa and Izmir provinces, western Turkey. Observed seroprevalence in dog is in agreement with the earlier reported 22.6% prevalence in Bulgaria [33], and 21% in Spain [12]. Our seroprevalence was higher than the prevalence reported in Bolivia (0.0%), Italy (0.0%), Sweden (3.9%) and The Netherlands (17%) [10, 16]; whereas it was lower than reported seropositivity in Germany (35.5%), Croatia (40%), Slovakia (45.3%) and Czech Republic (53.7%), using ELISA or IFA or indirect hemagglutination (IHA) methods [2].

Lyme disease in horses has been reported in several countries, with seropositivity ranging from very low to 68% [6, 15, 26]. The low frequency of seropositivity found in the present study might be due to the low levels of tick infestation in the racing horses from the studied area. Egenvall *et al.* [14] reported approximately similar seroprevalence (6.8%) to *B. burgdorferi* in horses in Sweden. Seropositivity obtained in horses in Monterrey, Mexico (34%), Wisconsin (63%) and Connecticut (84%), USA was much higher [15, 26]. On the other hand, Cohen *et al*. [11], found a 0.2% infection rate in horses in Texas. *B. burgdorferi* primarily causes recognizable signs of disease in horses [19, 29]. However, serosurvey in northeastern regions of the USA endemic for Lyme disease reported 14–24% seropositive horses, wherein only 10% of the seropositive subjects developed clinical signs [1]. In the present study, 18 (6.0%) out of 300 horses were seropositive with no clinical signs, this indicates the rare incidence of clinical borreliosis in horses.

Observed seropositivity in sick dogs was slightly greater than in healthy dogs (25% *vs.* 20.5%). In the study, the major clinical symptoms in seropositive dogs were: skin manifestations, vomiting, urinary tract infection and anemia. However, the common symptoms in dogs, such as coughing, heart failure and lameness, were not recorded frequently in the study. The erythema migrans that often develops in human cases is rarely reported in dogs. However, in the study, there was a significant association ($p < 0.01$) between seropositivity and skin manifestations in dogs. This finding most probably indicates that *B. burgdorferi* sero-positivity acts as a proxy indicator for animals that have recently had a tick bite, and it is the tick bite that causes the skin problems. Moreover, 2 out of 16 seropositive dogs with skin disorder had high serum titer (OD > 3.0), suggesting the involvement of *Borrelia*. Thirty-two seropositive dogs (20.5%), presented here, without clinical signs may have a subclinical infection. This is in agreement with Bushmick [1] and Fritz and Kjemtrup [15], suggesting that not all infected dogs develop clinical signs of Lyme borreliosis. In parallel, we found that antibody titers in seropositive healthy or sick dogs were not significantly different. Amongst 244 dogs with clinical problems, 61 (25%) were found to be seropositive and 18 (29.5%) had high titers (OD > 3.0), probably suggesting *Borrelia* as the causative agent for current clinical problems (Tab. 1). This is in line with the study of Magnarelli and Fikrig [23], who reported that dogs with signs of canine borreliosis have

stronger antibody responses to *Borrelia* than infected dogs without such signs.

Although breed predisposition has not been reported in dogs, we found that mixed breed was predominantly affected. The majority of mixed dogs were outdoor (stray) dogs and had a higher possibility of exposure to the infected ticks; this could be a possible explanation for the higher seroprevalence in these animals.

In conclusion, the occurrence of anti-*Borrelia* antibodies in dogs and horses provinces suggests that veterinarians should pay attention to this disease in their clinical practice and include it within the differential diagnosis. On the methodological side of this study, ELPAGA and BAT found equally sensitive assays for detection of anti-*Borrelia* antibodies. However, BAT can be used more effectively in animals – especially in game and free living where species specific conjugates are not available.

Acknowledgements

The work was supported by Grants Nos. 1/1354/04, VEGA 1/3239/06, AV/1104/2004 and AV4/0106/06 of the Slovak Republic.

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