VETERINARSKI ARHIV 70 (1), 39-45, 2000

Serologic studies of canine Lyme borreliosis in the Zagreb area (Croatia)

Nenad Turk[1]*, Albert Marinculić[2], and Zvonko Modrić[1]

[1]*Department of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*

[2]*Department of Parasitology and Invasive Diseases, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*

TURK, N., A. MARINCULIĆ, Z. MODRIĆ: Serologic studies of canine Lyme borreliosis in the Zagreb area (Croatia). Vet. arhiv 70, 39-45, 2000.

ABSTRACT

Sera from 120 apparently healthy dogs, 74 purebred, 46 mixed-bred, 57 females and 63 males in the Zagreb area (Croatia) were examined by enzyme-linked immunoadsorbent assay (ELISA) for antibodies to *Borrelia burgdorferi*. During triple i/v immunization of dog at 1, 7 and 14 day, on each occasion with 5 ml 1.4×10^7 cells/ml of *B. burgdorferi sensu stricto*, strain B31 ATCC 35210, a positive control serum was obtained at day 28 p.i., while negative control serum was obtained prior to immunization. The antigen for ELISA was prepared from the same bacterial strain sonicated on ice. The sonicate was quantified by sodium dodecyl sulphate polyacrilamid gel electrophoresis (SDS PAGE). IgG antibodies to *B. burgdorferi* were estimated in 6 (5%) samples of dog sera in titre ranging from 1:100 to 1:2000 (Optical density-OD=0.650 + 36.49, n=6). Antibodies to *B. burgdorferi* were found in two females and four males, two Labrador retrievers and in one German shepherd, a Hungarian viszla, one cocker spaniel, and a German wirehaired pointer at 2.5 to 8 years of age. Of 114 seronegative samples three samples were found to be very close to the margin of optical density that determined seropositive result (OD=0.562). Estimated seroprevalence to *B. burgdorferi* in dog sera suggested that dogs in the Zagreb area are infected with *B. burgdorferi* and that the Zagreb area is part of a wider Central European Lyme boreliosis endemic area.

Key words: *Borrelia burgdorferi*, dog, ELISA, Croatia

Introduction

Lyme borreliosis is primarily localized and subsequently a general infection in humans and animals caused by the spirochete *Borrelia*

* Contact address:

Mr. Nenad Turk, Department of Microbiology and Infectious Diseases, Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, 10000 Zagreb, Croatia, Phone and fax: 385 1 23 90 211, E-mail: nenad.turk@vz.tel.hr

ISSN 0372-5480
Printed in Croatia

burgdorferi sensu lato. Ixodes ticks transmit a causative agent. Clinical signs in humans include erythematous skin lesion associated with the tick bite, followed by arthritis, cardiologic, neurological and ophthalmologic abnormalities. A review of European literature reveals an inflammatory skin disease (acrodermatitis chronica atrophicans) described for the first time by the German physician Buchwald in 1883 (WEBER, 1986) and discovery of a migrating annular skin lesion that developed at the site of a tick bite (erythema migrans) by Afzelius in 1910 (WEBER, 1986). An outbreak of "juvenile rheumatoid arthritis" in the Connecticut community of Old Lyme was the first discovery of disease in the USA, described by STEERE et al. (1977).

Previously unrecognised spirochetes from the mid-gut diverticula of *Ixodes dammini* ticks were found by microscopic examination by BURGDORFER et al. (1982). BARBOUR (1984) succeeded in isolating and cultivating the micro-organism in modified Kelly's medium, now known as Barbour – Stoenner – Kelly II medium (BSK II). JOHNSON et al. (1984) described this micro-organism as a new species of the genus *Borrelia, Borrelia burgdorferi*.

B. burgdorferi was isolated from human blood by BENACH et al. (1983). This bacterial species was also isolated from the blood, spleen and kidney of white-footed mice (*Peromyscus leucopus*) and Eastern chipmunks (*Tamias striatus*) by ANDERSON et al. (1985), which suggested that feral rodents are a primary reservoir of infection. Arthritis of the carpal joint caused by *B. burgdorferi* was first reported in dogs by LISSMAN et al. (1984). Additionally, *B. burgdorferi* was recovered from blood, and a serum antibody titre was detected, respectively. *B. burgdorferi* was found in dog synovial fluid by darkfield microscopy and immunoperoxidase techniques by KORNBLATT et al. (1985). Analyses of serum samples of dogs in the USA, by an indirect fluorescent antibody method (IFA) and enzyme linked immunosorbent assay (ELISA) performed by MAGNARELLI et al. (1985, 1987), detected a high prevalence of serum antibodies to *B. burgdorferi* (66.5% and 76.3%). Antibodies to *B. burgdorferi* in dogs were also detected in Europe (KÄSBOHRER et al., 1994; CERRI et al., 1994; STEFANCIKOV et al., 1998; HOVIUS et al., 1999). Lyme borreliosis has been described in Croatia only in humans (BREITENFELD et al., 1986; BUREK et al., 1992; GOLUBIĆ et al., 1998). Considering the fact that northwest Croatia is a biotope of the *Ixodes ricinus* tick, the purpose of this study was to estimate the seroprevalence of *B. burgdorferi* in dogs in the Zagreb area.

Materials and methods

During 1998 in the Zagreb area (Croatia), 120 blood samples were obtained from vena cephalica antebrachii and vena saphena parva of apparently healthy dogs, 74 purebred and 46 mixed-breed, 57 females and 63 males, between 7 months and 15 years old.

One dog was intravenously (*i/v*) immunized three times at days 1, 7 and 14, on each occasion with 5 ml 1.4×10^7 cells/ml of *B. burgdorferi sensu stricto* strain B31 ATCC 35210 cultivated in Barbour-Stoenner-Kelly II (BSK II) medium.

The presence of antibodies was examined by the microscopic agglutination method (MA) and a positive control serum for enzyme linked immunosorbent assay (ELISA) was taken at day 28 *p.i.*

The antigen for ELISA was prepared from the same bacterial strain sonicated on ice. The sonicate was quantified by sodium dodecyl sulphate polyacrilamid gel electrophoresis (SDS PAGE). For sodium dodecyl sulphate polyacrilamid gel electrophoresis, proteins from whole-cell lysates (25 μg of protein per lane) were subjected to 12.5% sodium dodecyl sulphate polyacrilamid gel electrophoresis as described by LAEMMLI (1970). Gels were stained with Coomassie brilliant blue.

Optimum coating concentration of ELISA *B. burgdorferi* antigen was determined by checkerboard titrations of positive control serum against serial dilutions of antigen with carbonate buffer. Flat-bottom microtiter plates (Dynatech, Greiner, Germany) were coated overnight at 4 °C with 100 μl of the *B. burgdorferi* antigen diluted 1:500.

B. burgdorferi sensu stricto strain B31 ATCC 35210 was isolated from an *Ixodes dammini* tick in the United States (BURGDORFER et al., 1982).

After repeated washings, 100 μl of dog sera, diluted 1:100, were added and the plates were incubated for 30 min. at 37 °C. Plates were washed three times and horseradish peroxidase-conjugated rabbit anti-dog IgG (Sigma, Munich, Germany) was added to each well and the plates were incubated for 30 min. at 37 °C. After all washes were done, 100 μl of 5-aminosalicilic acid (Sigma, Munich, Germany) was added and after 30 min. of incubation at room temperature an optical density (OD) was determined photometrically at 405 nm. A test was considered positive if absorbance exceeded the mean plus 3 SD of the absorbances of sera of negative population (GRODZICKI and STEERE, 1988). Dog sera with estimated *B. burgdorferi* antibodies were finally examined in serial dilutions from 1:500 to 1:4000.

Results

Throughout the immunization study with *B. burgdorferi* strain B31 ATCC 35210, dogs remained healthy and rectal temperature remained within physiologic limits. An IgG antibodies for *B. burgdorferi* at day 14 *p.i.* in titre 1:1000, 21. *p.i.* 1:2000 and 28. *p.i.* 1:4000 were detected by MA and ELISA.

Within a whole cell sonicate of *B. burgdorferi*, we were quantify by sodium dodecyl sulphate polyacrilamid gel electrophoresis (SDS PAGE), the presence of major proteins that are designated flagelin (41 kD), OspB (34 kD) and OspA (29 kD) (Fig. 1).

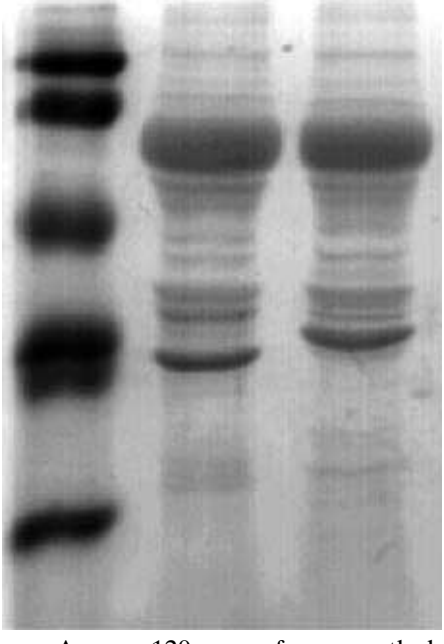

Fig. 1. A whole cell sonicate protein profile of *B. burgdorferi sensu stricto* strain B31 ATCC 35210 (SDS PAGE, Coomassie brilliant blue stain) St-Low Range Protein Standard, BIO RAD, immuno-dominant proteins flagelin 41 kD, OspB (outer surface protein) 34 kD and OspA 29 kD.

Among 120 sera of apparently healthy dogs in the Zagreb area, IgG antibodies to *B. burgdorferi* were estimated in 6 (5%) samples of sera in titre, ranging from 1:100 to 1:2000 (ODp=0.650 + 36.49, n=6) (Table 1). Antibodies to *B. burgdorferi* were found in two females and four males at age 2.5 to 8 years, in a Hungarian viszla and a German shepherd in titre 1:500, in two Labrador retrievers and in one cocker spaniel in titre 1:1000, and in a German wirehaired pointer in titre 1:2000 (Table 2). Of 114 seronegative samples, three were found to be very close to the margin of optical density that determined seropositive result (OD=0.562).

Table 1. Seroprevalence of antibodies to *B. burgdorferi* in 120 dog sera in the Zagreb area detected by ELISA

Result	N	%	IgG titre	Optical density OD $\overline{X}$	SD
Positive	6	5	>1:100	0.650	36.49
Negative	114	95	<1:100	0.392	56.91

Table 2. Titre of IgG antibodies to *B. burgdorferi* in seropositive dogs in the Zagreb area detected by ELISA

No.	Optical density - OD	IgG titre	Breed	Sex	Age (years)
1	0.667	1:500	Hungarian viszla	m	3.5
2	0.613	1:500	German shepherd	f	5.5
3	0.686	1:1000	Labrador retriever	m	8
4	0.654	1:1000	cocker spaniel	f	4.5
5	0.601	1:1000	Labrador retriever	m	4
6	0.686	1:2000	wirehaired pointer	m	2.5

Discussion and conclusions

Since dogs remained healthy throughout five weeks of immunization study after *i/v* exposure with *B. burgdorferi* strain B31 ATCC 35210 at 1, 7 and 14 days, and IgG antibodies to *B. burgdorferi* were detected by MA and ELISA at days 14, 21 and 28 *p.i.*, we considered that the strain used in the study lost pathogenicity and that it is suitable for immunization studies.

As shown by sodium dodecyl sulphate polyacrilamid gel electrophoresis (SDS PAGE) in whole cell sonicate obtained from *B. burgdorferi* strain B31 ATCC 35210, the presence of major proteins were confirmed, flagelin 41 kD, OspB 34 kD and OspA 29 kD which are

immunodominant and prove our sonicate successfully utilizable in the ELISA test.

The detected 6 (5%) seropositive dogs in our study with IgG antibodies in titre ranging 1:100 to 1:2000 indicate that the seroprevalence for canine Lyme borreliosis in the Zagreb area exists, considering the fact that we are on the border of a Central European endemic area.

Among the 114 sera considered to be negative, optical density in three sera were very close to the optical margin value for positive (OD=0.562), which raises the question as to whether those dogs were also infected.

In order to obtain a better insight into the epizootiology of Lyme borreliosis, further studies will be carried out to estimate the seroprevalence of Lyme borreliosis in a greater population of dogs and other animals in different regions in Croatia, particularly in those with suitable geoepizootiologic conditions for Lyme boreliosis.

Acknowledgments
The authors are grateful to Siegfried Brehm, D.Sc. of Landesuntersuchungsamt für das Gesundheitswesen Südbayern who in 1990 kindly provided the *B. burgdorferi* strain B31 ATCC 35210.

References

ANDERSON, J. F., R. C. JOHNSON, L. A. MAGNARELLI, F. W. HYDE (1985): Identification of endemic foci of Lyme disease: Isolation of *Borrelia burgdorferi* from feral rodents and ticks (*Dermacentor variabilis*). J. Clin. Microbiol. 22, 36-38.

BARBOUR, A. G. (1984): Isolation and cultivation of Lyme disease spirochetes. Yale J. Biol. Med. 57, 521-526.

BENACH, J. L., E. M. BOSLER, J. P. HANRAHAN, J. L. COLEMAN, G. S. HABICHT, T. F. BAST, D. J. CAMERON, J. L. ZIEGLER, A. G. BARBOUR, W. BURGDORFER, R. EDELMAN, R. A. KASLOW (1983): Spirochetes isolated from the blood of two patients with Lyme disease. N. Engl. J. Med. 308, 740-742.

BREITENFELD, V., N. MAKEK, T. MARETIĆ, A. BEUS (1986): Erythema migrans after tick-bite. Giorn. Mal. Inf. Parass. 38, 666-669.

BURGDORFER, W., A. G. BARBOUR, S. F. HAYES, J. L. BENACH, E. GRUNDWALD, J. P. DAVIS (1982): Lyme disease - a tick borne spirochetosis? Science. 216, 1317-1319.

BUREK, V., LJ. MIŠIĆ-MAJERUS, T. MARETIĆ (1992): Antibodies to *Borrelia burgdorferi* in various population groups in Croatia. Scand. J. Infect. Dis. 24, 683-684.

CERRI, D., R. FARINA, E. ANDREANI, R. NUVOLONI, A. PEDRINI (1994): Experimental infection of dogs with *Borrelia burgdorferi.* Res. Vet. Sci. 57, 256-258.

GOLUBIĆ, D., S. RIJPKEMA, N. TKALEC MAKOVEC, E. RUŽIĆ (1998): Epidemiological, ecologic and clinical characteristics of Lyme borreliosis in northwest Croatia. Acta Med. Croat. 52, 7-13.

GRODZICKI, R. L., A. STEERE (1988): Comparison of immunobloting and indirect enzyme-linked immunosorbent assay using different antigen preparations for diagnosing early Lyme disease. J. Infect. Dis. 157, 790-797.

HOVIUS, K. E., S. G. RIJPKEMA, P. WESTERS, B. A. VAN DER ZEIJST, F. J. VAN ASTEN, D. J. HOUWERS (1999): A serological study of cohorts of young dogs, naturally exposed to Ixodes ricinus ticks, indicates seasonal reinfection by *Borrelia burgdorferi* sensu lato. Vet. Quart. 21, 16-20.

JOHNSON, R. C., F. W. HYDE, G. P. SCHMIDT, D. J. BRENNER (1984): *Borrelia burgdorferi* sp. nov.: etiological agent of Lyme disease. Int. J. Syst. Bacteriol. 34, 496-497.

KÄSBOHRER, A., G. LIEBISCH, A. SCHÖNBERG, A. LIEBISCH (1994): Serodiagnostik der Lyme Borreliose: Entwicklung und Bewertung eines Testsystems für epidemiologische Untersuchungen beim Hund. Dtsch. Tierärztl. Wschr. 101, 476-481.

KORNBLATT, U. K., P. H. URBAND, A. C. STEERE (1985): Arthritis caused by *Borrelia burgdorferi* in dogs. JAVMA 186, 960-964.

LAEMMLI, J. F. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

LISSMAN, B. A., E. M. BOSLER, H. CAMAY, B. G. ORMISTAN, J. L. BENACH (1984): Spirochete-associated arthritis (Lyme disease) in a dog. JAVMA 185, 219-220.

MAGNARELLI, L. A., J. F. ANDERSON, A. F. KAUFMANN, L. L. LIEBERMAN, G. D. WHITNEY (1985): Boreliosis in dogs from southern Connecticut. JAVMA. 186, 955-959.

MAGNARELLI, L. A., J. F. ANDERSON, A. B. SCHREIER, C. M. FICKE (1987): Clinical and serological studies of canine borreliosis. JAVMA 191, 1089-1094.

STEERE, A. C., S. E. MALAWISTA, D. R. SNYDMAN, R. E. SHOPE, W. A. ANDIMAN, M. R. ROSS, F. K. STERLE (1977): Lyme artritis: an epidemic of oligoarticular arthritis in children and adults in three Connecticut communities. Arthritis Rheum. 20, 7-17.

STEFANCIKOV, A., G. TRESOV, B. PETKO, I. SKARDOV, E. SESZTAKOV (1998): ELISA comparison of three whole-cell antigens of *Borrelia burgdorferi* sensu lato in serological study of dogs from area Košice, eastern Slovakia. Ann. Agric. Environ. Med. 30, 5, 25-30.

WEBER, K. (1986): Remarks on the infectious disease caused by *Borrelia burgdorferi*. Zbl. Bakt. Hyg. A. 263, 206-208.

Received: 7 March 2000
Accepted: 28 March 2000

TURK, N., A. MARINCULIĆ, Z. MODRIĆ: Serološko istraživanje lajmske borelioze u pasa s područja grada Zagreba u Hrvatskoj. Vet. arhiv 70, 39-45, 2000.

SAŽETAK

Imunoenzimnim testom (ELISA) pretraženo je 120 uzoraka klinički zdravih pasa, 74 čistokrvna, 46 križanih, 57 ženki i 63 mužjaka područja grada Zagreba na prisutnost protutijela za *B. burgdorferi*. Trokratnom *i/v* imunizacijom psa 1., 7. i 14. dana s po 5 ml kulture *B. burgdorferi sensu stricto* soj B 31 ATCC 35210 u količini $1,4 \times 10^7$ bakterija/ml dobiven je 28. dan od početka imunizacije pozitivni kontrolni serum, a negativni je uzet prije imunizacije. Od istog soja načinjen je ultrazvučno lizirani antigen za imunoenzimni test (ELISA) čiji je proteinski sastav određen elektroforezom u poliakrilamidnom gelu uz dodatak natrijevog dodecilsulfata (sodium dodecyl sulphate polyacrilamid gel electrophoresis - SDS PAGE). U 6 (5%) uzoraka seruma pasa ustanovljen je titar IgG protutijela od 1:500 do 1:2000 (ODp=0,650 + 36,49, n=6) u dvije kuje i četiri psa u dobi od 2,5 do 8 god., u dva labradora i po jednog njemačkog ovčara, mađarske vižle, koker španijela i njemačkog oštrodlakog ptičara. Od 114 seruma u kojih apsorpcijska vrijednost nije prelazila graničnu apsorpcijsku vrijednost za pozitivne (OD=0,562) u tri seruma apsorpcijska vrijednost je bila blizu granične apsorpcijske vrijednosti. Ustanovljena IgG protutijela za *B. burgdorferi* u serumima pasa ukazuju da psi područja grada Zagreba bivaju inficirani bakterijom *B. burgdorferi* i da je to područje dio šireg srednjeeuropskog endemskog žarišta lajmske borelioze.

Ključne riječi: *Borrelia burgdorferi*, pas, ELISA, Hrvatska