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

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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 1.4×107 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 borreliosis 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

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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 carp 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 antebracli 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 $1.4 \times 10^7$ cells/ml of \textit{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 \textit{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 \textit{B. burgdorferi} antigen diluted 1:500.

\textit{B. burgdorferi sensu stricto} strain B31 ATCC 35210 was isolated from an \textit{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-aminosalicylic 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 \textit{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).

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).
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 geoeconomic conditions for Lyme borreliosis.

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**SAZETAK**

Imunoenzimnim testom (ELISA) pretraženo je 120 uzoraka klinički zdravih pasa, 74 čistokrvenih, 46 križanih, 57 ženkli 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 × 10⁷ 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 titer IgG protutijela od 1:500 do 1:2000 (ODp=0,650 ± 36,49, n=6) u dvije kuje i četiri pasa u dobi od 2,5 do 8 god.; u dva labradora i po jednoj njemačkoj ovčarci, mađarskoj viški, koke srpskome i njemačkog oštrodiokog ptitčari. Od 114 seruma u kojima apsorpcijska vrijednost nije prelazila graničnu apsorpcijsku vrijednost (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