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The diagnose of *Borrelia afzelii* infections in dogs

Rozpoznawanie zakażeń *Borrelia afzelii* u psów

Summary. The aim of this study was to recognize an etiological factor of diseases with symptoms of lameness and subcutaneous tissues oedema, which occurred in 4 dogs after invasions of the ticks. The serological examinations [ELISA and Western blot] were done. In the samples of all 4 sera a presence of specific anti-*Borrelia afzelii* antibodies (IgG) were detected. The results of serological examinations, and the reaction of sick animals on tetracycline therapy showed, that in all four cases an etiological factor of the diseases were spirochetes *Borrelia afzelii*. In the light of the research, veterinary practitioners should keep in mind the presence of Lyme disease in dogs in Poland and include it in differential diagnosis for lameness.

Key words: *Borrelia burgdorferi*, ELISA, Western blott, tetracycline.

INTRODUCTION

Lyme disease in dogs (borreliosis) is a systemic, multiorgan disease caused by spirochetes *Borrelia burgdorferi* belonging to *Spiorchetaceae* [Font *et al*. 1995]. It occurs endemically and is borne by *Ixodes* type ticks, particularly by *Ixodes ricinus* [Wodecka and Skotarczak 2000, Cisak *et al*. 2005, Foley *et al*. 2007]. In the course of the disease, the infected animals can show fever, apathy, arthritis [Appel *et al*. 1993], kidney damage [Grauer *et al*. 1988, Reusch *et al*. 1994], meninigitis, encephalitis, neuritis [Chang *et al*. 2001] and myocarditis [Breitschwerdt 1996]. Clinical symptoms do not occur in all cases of infected dogs; it is estimated that in endemic areas merely 5–10% out of 75% seropositive animals have shown clinical symptoms [Greene 1998]. The situation might be explained by the fact that some of the positive serological findings in animals result from non-pathogenic strain infection or a scant dose of *B. burgdorferi* cells infecting host with a well-functioning immune system.

Establishment of Lyme disease diagnosis is difficult and requires the presence at the same time at least four elements, that is clinical symptoms have to be accompanied by positive antibodies titre, together with tick exposure and a positive reaction to antibiotic therapy. Demonstration of the bacteria by culture techniques or detecting its presence in tissues by microscopic methods is very difficult due to a small number of bacterial cells in an infected organism. Nevertheless, the highest probability of isolation borreliae in skin biopsy appears to be at the onset of the disease, then after having administered antibiotic therapy, the PCR is the method of choice

The aim of the research was to determine the cause of the disease in four dogs, which had contact with ticks (September–November 2006).

MATERIAL AND METHODS

Cases description

The four dogs with clinical signs indicating of borreliosis naturally exposed to ticks were delivered to Clinic of Infectious Diseases Veterinary Faculty in Lublin. All the animals came from households located near forest, in the area where borreliosis occur endemically; they were not vaccinated against spirochetes infection. The clinical examination revealed in two German shepherd dogs symptoms of fever, strong apathy and lack of apetite. In the case of one pointer, high fever was observed and scrotum oedema, which expanded to subcutaneous tissue on the inner surface of thighs. In the case of the fourth dog – crossbreed, clinical examination revealed pyrexia (body temperature was slightly raised above physiological standards), apathy, lack of apetite, left ankle joint oedema with minor lameness and scrotum skin oedema. The clinical examination was followed by blood sampling for laboratory tests.

The blood samples were taken from cephalic vein into 2 tubes; one with EDTA for PCR test and hematological and microscopic examinations of blood smears, the second one without EDTA for ELISA and Western-blotting tests. The second set of blood samples for serological examination was taken 4 weeks later. In the differential diagnosis babesiosis, borreliosis and ehrlichiosis were considered

Treatment. Oxytetracycline (Oxywet Biowet Puławy) injection was administrated i.m twice daily (7 mg/kg), then replaced by doxycycline administered per os (10 mg/kg) within the period of three weeks. Additonally single dexamethasone (Dexafort Intervet) injection s.c. (0,15 mg/kg) was applicated to sick animals.

Molecular tests

PCR for *Babesia* and *Ehrlichia*. DNA was extracted from EDTA-anticoagulated whole blood using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) or the Blood Mini DNA isolation kit (A&A Biotechnology, Gdańsk, Poland) according to manufacturer instructions. The amplification of *B. canis canis* DNA through PCR was performed using the forward primer BAB GF2 (5'-GTC TTG TAA TTG GAA TGA TGG-3'), and the reverse primer BAB GR2 (5'-CCA AAG ACT TTG ATT TCT CTC-3'), which amplify a 559-bp region of the 18S rRNA gene of *B. canis canis* [Adaszek and Winiarczyk 2008].

Briefly, each reaction mixture (50 μL) contained 100 μM of each dNTP, 1.6 mM of $MgCl_2$, 0.25 μM of each primer, 2.5 U of *Taq* DNA polymerase, and 5 μL of DNA template. PCR amplification was performed using a programmable thermal cycler (Biometra, Goettingen, Germany) with the following program: an initial denaturation at 92°C for 2 minutes, 50 cycles of denaturation at 92°C for 60 seconds, annealing at 52°C for 60 seconds, and extension at 72°C for 90 seconds, followed by a final extension at 72°C for 5 minutes. Positive and negative controls were included in all amplifications. PCR results were evaluated by agarose gel (1%) electrophoresis stained with ethidium bromide in parallel with a 100-bp DNA ladder (Gibco/BRL, Gaithersburg, MD, USA).

The amplification of *Ehrlichia spp* DNA through PCR was performed using the forward primer EHR 521 (5'-TGT AGG CGG TTC GGT AAG TTA AAG-3'), and the reverse primer EHR 747 (5'-GCA CTC ATC GTT TAC AGC GTG-3'),which amplify a 247-bp region of the 16S rRNA gene of *Ehrlichia spp.*

Each reaction mixture (25 μL) contained 200 μM of each dNTP, 1.6 mM of $MgCl_2$, 0.125 μM of each primer, 2 U of *Taq* DNA polymerase, and 7,5 μL of DNA template. PCR amplification was performed with the following program: an initial denaturation at 92°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 45 seconds, followed by a final extension at 72°C for 5 minutes. Positive and negative controls were included in all amplifications. PCR results were evaluated by agarose gel (1%) electrophoresis stained with ethidium bromide in parallel with a 100-bp DNA ladder (Gibco/BRL, Gaithersburg, MD, USA) [Adaszek *et al*. 2009, in press].

Serological test for borreliosis

ELISA test. *B. afzelii* isolated from *Ixodes ricinus* in Poland were used as antigens, prepared by the method of Tresová *et al.* [1997]. The sera were examined by the modified ELISA test as described previously [Štefančíková *et al.* 2000]. Microplates were filled with 100 μl of respective antigen diluted in carbonate buffer at pH 9.6 and incubated overnight at 4°C. After washing three times with phosphate buffer (pH 7.2) containg 0.05% Tween 20, 100 μl of sera diluted in phosphate buffer with 0.05 % Tween 20 and 1% BSA were added to each well and incubated at 37°C for 30 min. After a triple washing of the plates, 100 μl of respective peroxidase conjugates (Sigma Antibody IgG A6792) were added per well. After 30 min of incubation and 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 working dilution of antigens and conjugates was estimated by the box titration. Sera from dogs that proved positive, resp., negative in repeated titrations in ELISA were used as controls. Cutt-off was determined as a value 3 standard deviations above the mean optical density (OD) for negative serum samples [Adaszek *et al.* 2008].

Western blotting. Sonicated antigen *B. afzelli* was dissolved in sample buffer (containing β-mercaptoethanol as reducing agent), boiled for 5 min and subjected to SDS-PAGE (12.5% polyacrylamide gel) using the system of Laemmli [1970]. Low-range-molecular-mass standards (BioRad) were used in gel.

Electrophoresis was carried out at constant current of 25 mA for 1 hour. Proteins separated on gel were then transferred to a nitrocellulose membrane using the system of Towbin *et al.* [1979]. The nitrocellulose membrane was cut into 3 mm wide strips. One part of each membrane was stained with amidoblack dye, to assess the efficiency of transfer, the other one was used to react with dogs sera. The strips were blocked for 1 hour at a room temperature with 5% non fat milk in BBS buffer, pH 8.2 (10 mM H_3BO_3, 25 mM $NA_2B_4O_7$ and 75 mM NaCl). The membranes were incubated for 2 h with dogs' sera diluted in 3% non fat milk-BBS. After three washes in 3% milk-BBS for 5 min, the strips were treated for 1 h with anti-dog IgG peroxidase conjugate (Sigma Antibody IgG A6792) diluted in 3% milk-BBS. After two additional washes for 15 min, the strips were developed by the addition of a freshly prepared solution of 0.075% (w/v) 4-chloro-1-naphtol (Sigma), 0.06 % hydrogen peroxide and 25% methanol in Tris buffer (pH 6.8). The reaction was stopped by washing the strips with distilled water [Štefančíková *et al.* 2008].

RESULTS AND DISCISSION

The observed clinical symptoms in dogs from eastern Poland, where the etiological factors of tick-born diseases occur endemically [Cisak *et al.* 2005, Winiarczyk *et al.* 2007, Adaszek and Winiarczyk 2007] indicated borreliosis in the animals under the study. To exclude the potential presence of *Babesia* and *Ehrlichia* organisms in erythrocytes and leucocytes respectively, microscopic examination of blood smear stained by Giemza method was conducted and a PCR amplification was done in order to detect their genetic material. Neither microscopic examinations of blood smears nor PCR method produced evidence of protozoans or *Rickettsia* in the examined material. No leucopenia or thrombocytopenia was discovered, which often accompany babesiosis and ehrlichiosis [Adaszek *et al.* 2009] however in all animals a hematologic examination revealed leucocytosis (Tab. 1).

Table 1. Results of hematological examination in dogs
Tabela 1. Wyniki badań hematologicznych przeprowadzonych u chorych psów

Parameters Badany parametr	German shepherd male Owczarek niemiecki samiec	German shepherd female Owczarek niemiecki samica	Pointer Pointer	Crossbreed dog Mieszaniec
RBC (10^6/mm^3)	8.12	7.45	5.88	7.82
Ht%	53.1	46.5	39.4	48.4
WBC (10^3/mm^3)	16.8	15.5	14.4	14.1
Hb g/dl	18.7	16.4	15.6	16.0
PLT (10^3/mm^3)	434	340	295	303

By means of ELISA test in which *Borrelia afzelii* cells were used as an antigen, an increased level of antibodies for the bacteria was revealed in all the dogs' sera. Absorbance value measured by wave 492 nm during the first examination ranged from 2,124 to 3,024. Due to a possibility of cross reactions between *B. burgdorferi* and other bacteria,

especially non-pathogenic spirochetes of *Borrelia* and *Leptospira,* the findings had to be confirmed by Western blott method. Antibodies contained in the examined sera reacted with protein fractions of 30, 33, 44, 56, 64, 68 kDa. The presence of electrophoretic bands of 56, 44, 33 kDa *B. afzeli*, which most probably represent protein p58, flagellin periplasmatic protein *B. burgdorferi* clearly indicate a natural infection. Molecular mass of protein fractions reacting in the Western blott test in our study differ slightly from those found in most papers concerning the issue [Greene 1998]. It mainly depends on the antigen which is used in individual research. In the present study it was *B. afzelii* strain isolated from ticks caught in area of eastern Poland, which could have differed from standard strains of *B. burgdorferi* routinely used in most of the investigations. For the same reason, a fraction of approximate of 22 kDa corresponding to surface protein OspC, did not appear on the electrophoregram. It is worth noting here, that this specific protein undergoes expression only in higher temperatures in host organisms during the course of infection and does not occur in spirochetes cells multiplied on artificial media or isolated from ticks [Greene 1998].

Table 2. Absorbations values of dogs sera in ELISA test
Tabela 2. Wartości absorbancji próbek surowic badanych testem ELISA

Dogs Psy	Specimens taken at the admission of the dog to the clinic (day 0) Próbki pobrane do badań w dniu zgłoszenia psów do kliniki (dzień 0)	Specimens taken after three weeks therapy Próbki pobrane do badań po trzytygodniowej terapii
German shepherd (male) Owczarek niemiecki (samiec)	2.847	2.050
German shepherd (female) Owczarek niemiecki (samica)	3.024	2.344
Pointer	2.124	1.884
Crossbreed dog Mieszaniec	2.766	2.010

The antibiotic and corticosteroid therapy resulted in major improvement led to a marked regression of clinical symptoms, inner body temperature came back to normal, apathy regressed, appetite returned. A follow-up serological examination four weeks later done by ELISA test revealed a significant drop in antibodies level in blood serum compared with the first examination. Absorbation of sera samples taken from the sick dogs significantly dropped and ranged from 1,884 to 2,244 (Tab. 2) at the time. The disappearance of clinical signs followed by treatment with tetracycline combined with the drop of specific antibodies titer between acute and convalescent serum samples is indicative of recent infection with *Borrelia* organisms. Suspicion of that infection is strongly supported by the fact of exposition of the dogs to ticks in the endemic area for tick-born diseases.

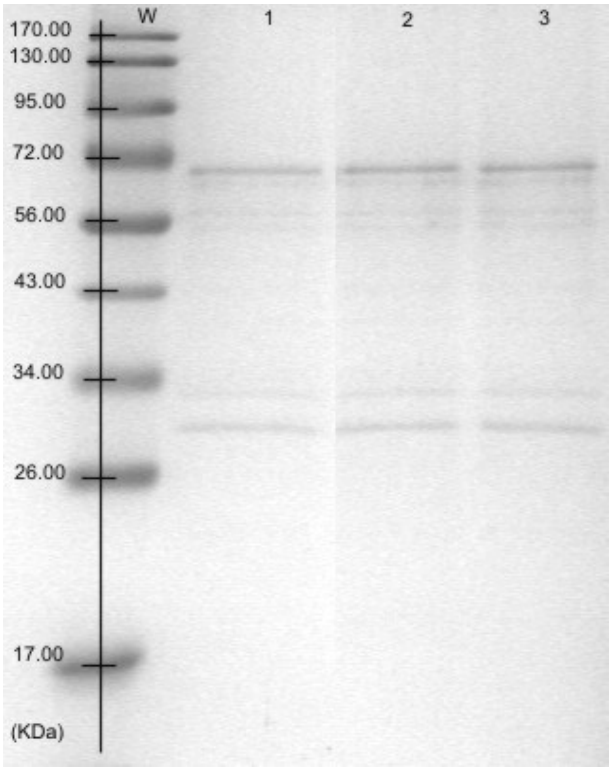

Fig. 1. Immunoblot of dogs sera with local strain *Borrelia afzelii*
Rys. 1. Wyniki badania immunoblotingu surowic z zastosowaniem lokalnego szczepu *Borrelia afzelii*

In the light of the research, veterinary practitioners should keep in mind the presence of Lyme disease in Poland and include it in differential diagnosis for lameness in dogs especially when there are associated systemic signs and a history of exposure to ticks.

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Streszczenie. Celem pracy było określenie przyczyny choroby u 4 psów, przebiegającej z objawami kulawizn oraz obrzęku tkanki podskórnej po kontakcie z kleszczami. Badaniami serologicznymi (ELISA i Western blot) w próbkach surowic pobranych od wszystkich zwierząt wykazano obecność sprzeciwiał swoistych dla *Borrelia afzelii* (IgG). Wyniki testów serologicznych oraz pozytywna odpowiedź chorych psów na terapię tetracyklinami wskazują, iż przyczyną choroby było zakażenie krętkami *Borrelia afzelii*. Uzyskane wyniki wskazują na konieczność uwzględnienia choroby z Lyme w diagnostyce różnicowej kulawizn u psów.

Słowa kluczowe: *Borrelia afzelii*, ELISA, Western blott, tetracykliny