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Borrelia burgdorferi in ticks and dogs in the province of Vojvodina, Serbia[1]

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Summary:

Lyme disease is a tick borne zoonotic infection, caused by *Borrelia burgdorferi* s.l. bacteria. For the transmission of the disease, the presence of ticks is a prerequisite. Lyme borreliosis mostly occurs in people and dogs, but it may occur in other animals. Ticks which carry *B. burgdorferi* s.l. in Serbia are of the *Ixodes ricinus* specis. In Serbia, Lyme disease was detected for the first time in the late '80-es. In dogs, clinical symptoms may occur even months after a tick bite, and include weakness, lymphadenopathy, fever, lameness, arthritis, etc. In our survey, we have observed tick and dog populations in the province of Vojvodina (northern part of Serbia). *I. ricinus* ticks were collected and examined for the presence of *B. burgdorferi* s.l. in several chosen locations. In addition, blood samples were collected from house dogs and pets from the same locations, and analyzed for the presence of antibodies specific for *B. burgdorferi* s.l. The results showed a mean infection of ticks of 22.12 %, and a mean seroprevalence of Lyme disease in dogs of 25.81 %. We conclude that in Vojvodina there is an actual risk of Lyme borreliosis for other animals and humans, because of the persistence of *B. burgdorferi* s.l. in both tick and dog populations.

KEY WORDS: *Borrelia burgdorferi*, Lyme borreliosis, diagnosis, *Ixodes ricinus*, tick, dog.

Résumé : *Borrelia burgdorferi* chez les tiques et les chiens de la province de Voïvodine, Serbie

La maladie de Lyme est une zoonose transmise par les tiques, due à l'infection par des bactéries Borrelia burgdorferi *s.l. La présence de tiques est nécessaire à la transmission de la maladie. La borréliose de Lyme touche l'homme et le chien, mais elle peut aussi survenir chez d'autres animaux.* Ixodes ricinus *est la tique vectrice de* B. burgdorferi *s.l. en Serbie, où la maladie de Lyme a été détectée pour la première fois à la fin des années 1980. Chez le chien, les symptômes cliniques, qui peuvent survenir des mois après une morsure de tique, sont une asthénie, une lymphadénopathie, de la fièvre, une boiterie, de l'arthrite, etc. L'étude a porté sur les populations de tiques et de chiens de la province de Voïvodine, au nord de la Serbie. La présence de* B. burgdorferi *s.l. a été recherchée chez des* I. ricinus *collectés dans trois zones prédéterminées. La présence d'anticorps spécifiques dirigés contre* B. burgdorferi *a été recherchée dans des échantillons de sang de chiens et d'autres animaux de compagnie des trois mêmes zones. La prévalence moyenne de l'infection chez les tiques est de 22,12 % et la séroprévalence moyenne de la maladie de Lyme est de 25,81 % chez les chiens. En conclusion, il existe dans la province Voïvodine un risque de borréliose de Lyme chez l'animal et l'homme en raison de la présence persistante de* B. burgdorferi *s.l. tant dans les populations de tiques que de chiens.*

MOTS CLÉS : *Borrelia burgdorferi, borréliose, maladie de Lyme, diagnostic, Ixodes ricinus, tique, chien.*

Lyme disease is a vector borne disease, which has been known for the past 40 years. In Serbia, it has been recognized some 30 years ago (Lako *et al.*, 1998). The disease can be found in humans and animals, and presence of ticks is a prerequisite for disease transmission.

In addition to humans, the disease can often be found in dogs and sometimes in other domestic animals. Lyme disease is a systemic, infectious zoonotic disease, caused by the spirochete *Borrelia burgdorferi* s.l. It is primarily transmitted by ticks from the Ixodidae family (Burgdorfer, 1989). In Serbia, as elsewhere in central Europe, Lyme disease is transmitted by *Ixodes ricinus* (Perng *et all.*, 1991) ticks (Fig. 1). Infection of the host occurs during the blood meal of a vector (tick), infected with a pathogenic strain of *B. burgdorferi* s.l. So far, 13 strains of *Borrelia burgdorferi* s.l. have been identified, three of which are pathogenic for humans and animals. These include *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii* (Barbour & Hayes, 1986). The distribution and presence of pathogenic genospecies of *B. burgdorferi* s.l. in ticks in Serbia, as well as their influence on clinical manifestations of Lyme disease in dogs, is yet to be evaluated.

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Ticks are mostly found in public parks, close to big cities, and their survival is determined by their ability to complete the entire life cycle, including the necessary hosts and developmental stages (Pejchalova, 2007). Lately, ticks have been found in children playgrounds, school and kindergarten yards and also in dog playgrounds within parks.

During the last five years, surveys showed that ticks in Serbia are infected with *B. burgdorferi* s.l. at a rate of 25-30 %, depending on the region (Milutinović *et al.,* 2004; Milutinović *et al.,* 2008, Cekanac *et al.*, 2009). Also, a number of clinical cases of Lyme disease was registered in humans and dogs (Dmitrović, 1996; Savić-Jevđenić *et al.,* 2008). Accurate data on the possible relation between the number of infected dogs and the percentage of ticks infected with *B. burgdorferi* s.l. within particular regions are still lacking. According to one study, the percentage of ticks infected with *B. burgdorferi* s.l. in the province of Vojvodina ranges from 25-28 % (Savić *et al.,* 2007). An analysis of ticks in selected urban regions performed during a three-year period revealed an infection rate of 25 % (Jurišić, 2008). In dogs, ticks are most likely to be found near the eye, exterior and interior surface of the ear, around the nose and neck and also on the back, under the front and back legs and between fingers (Skotarczak, 2007). After a natural infection of dogs with *B. burgdorferi* s.l. pathogenic strains, clinical signs are found in only 5 % of infected animals. The clinical signs include loss of appetite, weakness, lymphadenopathy, and fever. Later on, 2-5 months after the tick bite, intermittent lameness and mono- or oligoartritis may occur, which last up to a few weeks. Acute and subacute arthritis may develop, which can persist or reoccur even after treatment, and often expand to the chronic form of Lyme arthritis.

Since there are no pathognomonic clinical signs, diagnosis of Lyme disease in dogs relies on laboratory diagnostic procedures. Most often it is based on the detection of *B. burgdorferi* specific antibodies in the serum. A variety of serological methods may be used, including complement fixation (CF), ELISA, Western blot, immunofluorescence and immunochromatographic fast tests. A study performed on hunting dogs revealed a seroprevalence of 15-20 %, many of which did not show any clinical symptoms (Savić *et al.*, 2008). To interpret serological results, insight into the local epidemiological situation is thus very important (Littman, 2006).

MATERIAL AND METHODS

Ixodes ricinus ticks were collected in the field with a white flag of 1 m^2, from the meadow and forest vegetation during the tick season (end of March - beginning of June, and end of August - end of October). Ticks were picked during a three year period (2006-2008) within four regions of Vojvodina (Ada-Mol region, Novi Sad region, Fruška gora region and Novi Bečej region). The gender and life cycle stage were determined morphologically, and abdominal content examined for the presence of *B. burgdorferi* s.l. by dark field microscopy. The isolation of autochthonous strains of *B. burgdorferi* s.l. from ticks was performed in selective media BSK-H complete medium, with 6% rabbit sera (Zuckert, 2007). The tubes with selective media and inoculated tick content were incubated at 33 °C and checked every seven days by dark field microscopy for the growth of *B. burgdorferi* s.l. Genotyping of *Borrelia* isolates was done by PCR. For preparation of the samples, 1-3 ml of isolated *Borrelia* culture in BSK media was centrifuged for 5 minutes. Supernatant was removed and the sediment washed first in PBS pH 7,0 and then in distilled water. The samples were then cooked for 15 minutes in boiling water. After another centrifugation, the supernatant was moved to new tubes and then kept at -20 °C. The multiplication process was done by mixing 5 µl of prepared sample and 45 µl of PCR mix (5 µl 10 X concentrated buffer (Applied Biosystems), 1,5 mM $MgCl_2$, 100 µM of each dATP, dGTP, dCTP and TTP, 15 pmol of primer and 1,5 U *Taq* DNA polymerase. The "50bp DNA Ladder" was used as a marker of known molecular weight. PCR products were electrophoresed on 1.5 % agarose gel and stained with ethidium bromide. The order of primer nucleotides was taken from Kupier et al (Kupier *et al.*, 1994).

In addition, blood samples were drawn from 486 dogs living in the same region where the ticks were collected. Most of them were house dogs and pets, both males and females, of different breeds, from 2 to 14 years of age. Blood samples were analyzed by ELISA and Western blot for the presence of *B. burgdorferi* specific antibodies. ELISA (recomWell Borrelia canis IgG, Mikrogen, Germany) was performed according to the manufacturer's instructions. This test contains recombinant antigens for the detection of IgG antibodies against *B.*

Fig. 1 – *I. ricinus,* adults female (left) and male (right), Jurišić (2008).

burgdorferi s.l., *B. garinii*, *B. afzelii*. Western blot (recomBlot Borrelia Canis IgG, Mikrogen, Germany) was also performed according to the manufacturer's instructions. The antigens used in this test include the highly specific *B. burgdorferi* antigens p100, p39, p18, and p41 (flagelin), as well as the OspA and OspC outer surface proteins. OspC protein from all three genospecies can be found (*B. burgdorferi* s.l., *B. garinii* and *B. afzelii)*, and also the ones from two different strains of *B. garinii* (strain T25/OspA serotipe 7 and strain 20047/OspA serotipe 0), named as *B. garinii* 1 and *B. garinii* 2.

RESULTS AND DISCUSSION

During the three-year study period, a total of 1,224 ticks were collected from different locations in Vojvodina. The dominant species was *Ixodes ricinus*, accounting for 62 % (764 ticks) of all collected ticks. All ticks were analyzed for the presence of *B. burgdorferi* s.l. (Table I).

The seroprevalence of *B. burgdorferi* s.l. infected ticks in the territory of Vojvodina during the survey period (2006-2008) was 22.12 %, but ranged widely, from 11 % to 29 %, depending on location and year. In 2008, the seroprevalence of *B. burgdorferi* s.l. in ticks was 23.8 %, which is moderate compared to other European countries, where it ranges from 11-12 % in Spain and Portugal, 14,8 % in the Czech Republic and 19 % in Switzerland, to 45 % in some parts of Croatia and even to 60 % in the Lublin region in Poland (Pejchalova, 2007, Moran Cadenas *et al.*, 2007). The results showed that the distribution of infected ticks was not the same in the whole Province of Vojvodina. In 2008, there was one region (region of Novi Bečej) where none of the collected ticks were infected. In this particular region there were no seropositive dogs either.

Isolation of *B. burgdorferi* s.l. was attempted from tick pools, pooled according to gender and life cycle stage. From the total of 26 pools, the growth of spirochetes in the BSK-H media (Sigma) was noticed in four (15 %). These pools were named after the locations, or the marks of the locations where they were picked. In pool 1, 2 and 3 the growth of spirochetes was spotted after 14 days, whereas in the fourth pool, the growth began after 21 days. The final concentrations of isolated strains of *B. burgdorferi* s.l. were from 32-76 X 10^5 *B. burgdorferi* / ml culture for four different pools.

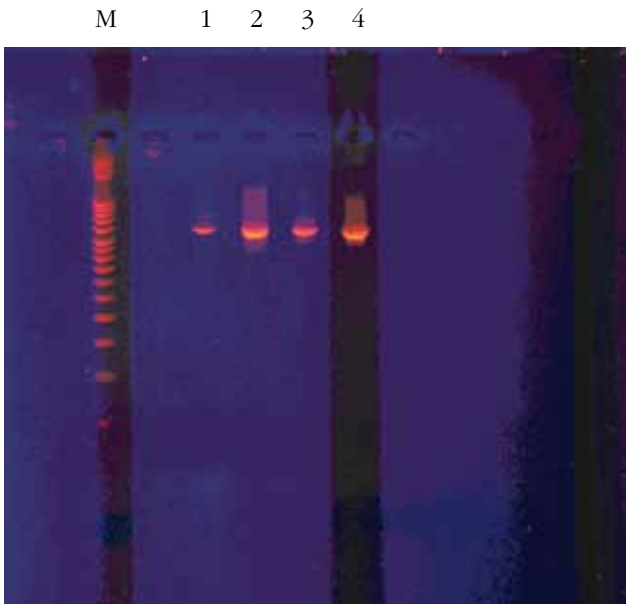

Fig. 2 – Species specific PCR of *Borrelia burgdorferi* sensu lato isolates; Lines: M, DNA size marker, 50 bp DNA ladder; 1 (tick pool 1), isolate amplified with primers specific for *Borrelia burgdorferi* sensu stricto; 2-4, isolates (Tick pool 2-4) amplified with primers specific for *Borrelia afzelii*.

B. burgdorferi s.l. is very difficult to culture, demanding in conditions, and bacteria undergo certain changes in order to adjust to *in vitro* growth. We therefore did not achieve growth of *B. burgdorferi* s.l. from each sample. It is known that the number of spirochetes in the tick sample and the type of media used, along with the aseptic conditions needed for working with borrelia, can very much influence *in vitro* growth (Ružić-Sabljić *et al.*, 2006).

Genotypization of *B. burgdorferi* s.l. isolates from *I. ricinus* ticks showed the following results:

- tick pool 1: *B. burgdorferi* sensu stricto (specific fragment 575bp);
- tick pool 2: *Borrelia afzelii* (specific fragment 591bp);
- tick pool 3: *Borrelia afzelii* (specific fragment 591bp);
- tick pool 4: *Borrelia afzelii* (specific fragment 591bp).

From the total of 486 dog blood samples obtained from dogs living in the regions where 11-29 % of ticks were infected with *B. burgdorferi* s.l., 25.5 % were found to

Year	Total No collected ticks	Total No & % of *I. ricinus*	No & % of adult females	No & % of adult males	No & % of nymphs	No & % of ticks infected with *B.b.* s.l
2006	386	232 (60 %)	109 (47 %)	102 (44 %)	21 (9 %)	44 (18.9 %)
2007	479	302 (63 %)	173 (57 %)	93 (31 %)	36 (12 %)	94 (19.6 %)
2008	359	230 (64 %)	90 (39 %)	119 (52 %)	21 (9 %)	55 (23.8 %)
TOTAL	**1,224**	**764 (62 %)**	**372 (49 %)**	**314 (41 %)**	**78 (10 %)**	**169 (22.12 %)**

Table I. – The annual number of collected ticks infected with *Borrelia burgdorferi* s.l. during the period 2006-2008.

	Total No	No (%) of positive samples to *B.b.* s.l by ELISA	No (%) of positive samples to *B.b.* s.l. by Western blot
Blood samples	486	124 (25.5 %)	127 (26.1 %)

Table II. – Dog blood sera analysis for the presence of specific antibodies against *Borrelia burgdorferi* s.l. by ELISA and Western blot test.

be positive for *B. burgdorferi* s.l. by ELISAand 26.1 % by Western blot (Table II).

The owners of pet dogs, especially in urban locations, are not sufficiently aware of the risk nor are familiar with the possible consequences of a tick bite, so they often do not use any products for prevention of tick bites. Also, owners of pet dogs are frequently poorly informed about the number of ticks infected with *B. burgdorferi* s.l. in their region and also about the risk these ticks represent for their dogs and themselves.

Abundant literature data report that the epidemiological profile of dog borreliosis represents a potential indicator of the risk for human borreliosis in a certain region (Skotarczak, 2007). Translated to the analyzed regions, the results of this study indicate that in Vojvodina there is a risk for human borreliosis. Tick control is a major measure for the prevention of borreliosis, and to that purpose tick repellents for pets and acaricides (amitraz, permetrin) for animals and the environment should be used, as well as prompt mechanical removal of the tick from the skin (Shaw, 2008).

The most friendly conditions for ticks are the regions between forests and meadows and they depend on geographic locations, altitude, flora and fauna and the presence of hosts. The most important factor that influences the survival of ticks in a region is air humidity. In mixed woods, the largest population of ticks is in places where humidity reaches 70-80 %. The survival of a tick in a certain region depends on the possibility to complete its life cycle (Pejchalova, 2007). In Northern Serbia, humidity can be very high, so active ticks were found even in November, when the outdoor temperature was below 8 °C, but the humidity was over 80 % (Jurišić, 2008).

Infection with *B. burgdorferi* s.l. is present in the tick population in Vojvodina, at an average rate of 22.12 %; however, the results showed it ranged from 11-29 %, depending on the year and region. In addition, infection with *B. burgdorferi* s.l. is present among the dog population in Vojvodina, at a rate of 25.5 % as detected by ELISA and 26.1 % as detected by Western blot. Among the dog population living in the region where ticks infected with *B. burgdorferi* s.l. were found, there are dogs with positive serological findings for Lyme borreliosis, even though none of the clinical signs of the disease can be seen. Thus, it may be concluded that in Vojvodina there is a real risk of Lyme borreliosis in humans, because of the persistence of the disease in both tick and dog populations.

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