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Discovering Lyme Disease in Ticks and Dogs in Serbia – Detection and Diagnostic Methods

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1. Introduction

Lyme disease is one of the infectious diseases discovered in the last three decades. It is a systemic, infectious and zooantropoic disease. Lyme disease is caused by spirochetes *Borrelia burgdorferi* s.l., and is primarily transferred via *Ixodes* ticks. Ticks that are vectors for Lyme disease in Europe and also in Serbia are from *Ixodes ricinus* species (Burgdorfer et all, 1989). Up to date the existence of 13 strains of *Borreliae burgdoerferi sensu lato*, is confirmed and only three of them are pathogens for humans and animals: *B. burgdorferi sensu stricto, B. afzelii* and *B. garinii*. After natural infections of dogs with pathogen strains of *B. burgdorferi* s.l. clinical symptoms are found in 5% of infected dogs. In most of the cases clinical symptoms are similar to a second stadium of Lyme disease in humans – anorexia, weakness, lymphadenopathia, increase of body temperature. Later, 2-5 months after a tick bite an intermittent lameness can be found.

There are no characteristic clinical symptoms and that makes diagnostic of Lyme disease quite a challenge. That is why laboratory diagnostic is necessary and sometimes the only way to correct diagnosis. Laboratory diagnostic is based on detection of specific antibodies against *B. burgdorferi* s.l. in blood serum and synovial fluid; isolation and detection of *B. burgdorferi* s.l. genome with molecular method.

During the last five years a certain percentage of ticks infected with *B. burgorferi* s.l. has been discovered (25 - 30% in different regions) in Serbia (Milutinović et al 2008, Cekanac et all 2009). Also, clinical cases of the disease are found in dogs and humans.

2. History

Lyme disease is actually present in many countries and only in Europe; over 19 countries have confirmed the existence of the disease (Burgdorfer, 1991). According to the literature data, several studies have been done in the country and in the surrounding countries in the domain of veterinary and human medicine on Lyme disease and on tick infections with *B.burgdorferi.* The first human case of Lyme borreliosis in USA was detected in 1975. From that time, numerous studies have been done in different countries and regions (Duncan et all, 2005). Prevalence for Lyme disease found in dogs in Alabama and North Carolina was rather low, even under 3%, while in New Jersey the prevalence was over 30% and in Wiskonsin, New York and Connecticut it was over 50% (Magnarelli et all 1988, Shulze et all 1987, Wright et all

1997). Since 1995. Until today, human cases of Lyme disease have been reported in 48 countries in USA. Sudies done in Europe have shown various data. In northwestern part of Croatia 45% of ticks were infected with *B.burgdorferi s.l.* (Golubić et all, 1998). In Lublin region,Poland, over 60% of ticks infected with causative agent of Lyme disease were found (Cisak et all, 2006.). Data for Switzerland, implicate that 19% of ticks were infected with *B.burgdorferi s.l.* (Moran Cadenas et all, 2007). In Portugal, during the past 15 years, cases of Lyme disease in humans were reported in 17 regions, from 20 regions in total, while prevalence in ticks was found from 11/35% (Lopes de Carvalho and Nuncio, 2006.). Research done in Spain resulted in similar data for prevalence of 11% in dogs and humans (Merino et all, 2000.), even though *I.ricinus* is not dominant population of ticks - only 12% of total tick population are *I.ricinus* ticks(Merino et all, 2005.). After the research was done in Check Republoc, 30% of ticks were found to be infected with B.burgdorferi s.l. (Hulinska et all 2007.).

In Serbia some research is done in the region of Belgrade, where 20-30% of ticks are infected with *B.burgdorferi*, in 2004, seroprevalence in ticks was 21,8 %(Milutinović et all, 2004; Čekanac et all, 2009) and in 2005, it was 17,5%. During a few years period (2001-2004) infection was found to be even 42% in the selected sample of ticks (Milutinović et all, 2008). In Resavica region, during 2007, infection of ticks was on average 33,6%. In Vojvodina region (northern part of Serbia), during a period from 2004-2007, 22-28% of infected ticks were found (Jurišić, 2005; Savić-Jevđenić et all, 2007). In Belgrade (capital of Serbia) Lyme disease was detected in 1987, for the first time (Dmitrović, Popović, 1993; Lako et all, 1998), and later on in the other parts of Serbia. In the autumn of 1989 in a daily newspaper an announcement from Prof. dr Boriša Vuković (at that time a Director of Epidemiology service, Medical faculty Novi Sad) was published: "If there was not AIDS, Lyme disease would surely be No 1 disease in its spreading and epidemiological value, among the new disease". Since December 1989, Lyme disease is obligatory for announcing if appears, in Serbia. From 1991 to 2004, 7774 people are registered for having Lyme disease. Of all those patients, 47,8% were from the territory of Belgrade and 23,7% of the patients were from Vojvodina region. Incidence for Vojvodina was from 0,4 in 1991, to 11,7 in 2004 (Epidemiological bulletin, 2005).

According to the data from Medical Clinical Center of Vojvodina, number of humans that had clinical symptoms of Lyme disease with laboratory conformation of diagnosis during the last 5 years was the following:

- 2006 28 patients;
- 2007 48 patients;
- 2008 54 patients;
- 2009 40 patients;
- 2010 53 patients;
- 2011 33 patients in the first 8 months;

In 2008, there were 50% more patients then in 2006 and then in the following period the annual number of patients was more or less the same. According to the report in Health and statistical yearbook of Serbia published by Institut for public health of Serbia „Dr Milan Jovanović Batut", the number of patients with Lyme disease in Serbia was in total 651 during 2007, of wich 456 were in central Serbia and 195 cases were in Vojvodina. Lyme disease cases make 97% of all vector borne diseases recorded in 2007 in Serbia and 99% in Vojvodina.

3. Ecology, epidemiology – life cycle of ticks and tranfer of the causative agent

The best conditions for tick's life cycle in Serbia can be found in the regions between woods and meadows and also between deciduous and evergreen forests. Geographical position of the localities, elevation, composition of flora and fauna, presence of the hosts, vectors and enough food for rodents represent the optimal conditions for maintenance and circulation of *B.burgdorferi* s.l. The most important factor that influences the quality of tick's life is air humidity. In mixed woods, the tick population is highest in the places where humidity reaches 70-80%. Little paths through woods, which are used by forest animals, are the places with a highest risk for humans and animals.

Picture 1. *I. ricinus*, adults female and male, Jurišić (2008)

Ticks, infected and not infected can often be found in urban parks with woods, very near to the big cities. Appearance of ticks in the cities seems to be connected to development of new parks and woods and also building of urban parts on the edge of a forest. The survival of ticks in certain localities is determined by a possibility to realize the whole life cycle of ticks with all the hosts needed and developing stages (Wall, Shearer, 1992; Pejchalova, 2007).

A dominant species of ticks that carry Lyme disease in Serbia is *Ixodes ricinus*. This tick has a two season phase in life cycle – spring and autumn phase. During the tick season, a change in prevalence of Lyme disease can be expected. Research done during the '90-ties, when *B. burgdorferi* s.l. was isolated for the first time in the region of Belgrade, until nowadays show that infection of ticks with *B. burgdorferi* s.l. is from 20-30%, with a mild trend of growth (Milutinović et all, 2004). For the region of Vojvodina, this percentage is from 25-28% (Jurišić, 2005; 2008; Savić –Jevđenić et all, 2007).

On the localities in the urban parts, ticks were mostly found in woods and parks with woods and the smallest number of ticks was found in landscaped parks, because the treatment against ticks was always performed there, during the spring period. Besides that, in woods and parks with woods the influence of mankind is less, so these places are rich in flora and fauna, compared to the localities in urban places. Based on the research done during the period from 2002–2004, author Jurišić concludes that far most common tick

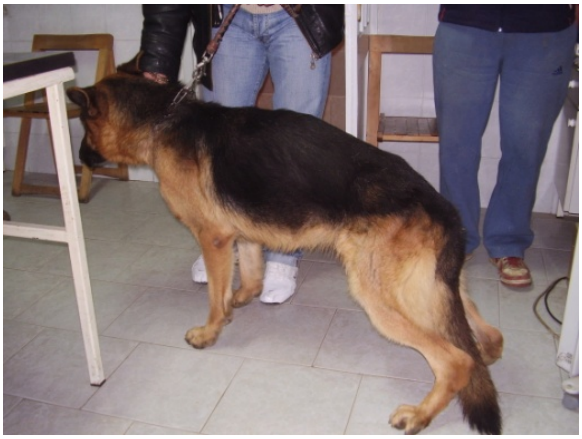
species, present in urban regions is *Ixodes ricinus* (Jurišić, 2005). Depending on the weather conditions, the presence of ticks was spotted until the beginning of October, in 2003, and in 2004 the tick activity was registered during the whole year, with the maximal periods – the last one in November when air temperatures were below 8^{o}C and relative air humidity over 85%. During this three year period, infection of ticks was analyzed in chosen localities of urban regions. In total 461 ticks from different locations was analyzed for the presence of *B. burgdorferi* s.l. spirochetes and mean infection of 25% in ticks was determined. In several chosen localities where no chemical treatment was ever used, the infection of ticks reached 29% and the lowest infection rate of 16% was determined in the urban parts of cities (Jurišić 2005; Jurišić, 2008).

Lindgren and Jaenson, while working on project about the influence of climate changes to the presence of Lyme disease, concluded that from the '80-ties, density and spreading of ticks has increased towards higher latitude and longitude. The climate changes announced for Europe will bring to spreading of Lyme disease to higher latitude and longitude. On the other hand in some regions where Lyme disease can be found now, when climate becomes too hot and dry for tick survival, Lyme disease might disappear from those regions. According to the prediction in the next 50 years, the climate in Europe will become milder, with higher winter air temperatures and longer vegetation period with higher risk of floods in the north of Europe and dry air in the southern parts of Europe. All of these elements can influence directly the number and distribution of ticks and their hosts in the region (Lindgren and Jaenson, 2004).

Pictures 2. and 3. –Woods near the urban region where infected ticks were found.

3.1 Pathogenesis and immunological response

Criteria for clinical diagnostic of Lyme disease in dogs are defined in 1992: anorexia, depression, malfunction of limbs or joints. After a natural infection clinical symptoms may appear even few months after the infection as lameness and joint swelling, which lasts for 3-4 days and then the symptoms may disappear and then reappear after a few weeks or months (Epidemiological bulletin, 2005). A research was done on hound dogs aiming to highlight that weather there are dogs without any clinical symptoms or those with typical clinical symptoms, total prevalence for Lyme borreliosis is 15-20% (Savić-Jevđenić et all, 2008).



Picture 4. German shepherd with lameness and positive serological finding for Lyme disease (Veterinary practice „Leo", Novi Sad, Lolić, 2008.)

In dogs there is no erythema migrans stage, but there can be skin leisure after tick bite. After natural infection with *B.burgdorferi* a renal malfunction can appear in form of glomerulonephritis and changes in renal tubule, but renal malfunction are not characteristic only for Lyme boreliosis. There are no changes in hematological and biochemical blood parameters in cases of Lyme disease (Dmitrović and Popović, 1993).

4. Diagnostic

Diagnostic of borreliosis is based on epidemiology, epizootiology, clinical symptoms, laboratory tests and reaction to antibiotic treatment (Skotarczak, 2007). Standard serology tests are based on detection of specific antibodies produced in dog's organism against *B. burgdoerferi* s.l. Many animals get into contact with *B. burgdorferi* s.l. and consequently antibodies appear, but there are no clinical symptoms. In Serbia there is no regular vaccination against Lyme borreliosis. Dogs suspicious for Lyme boreliosis have to have a history of tick bite, some clinical symptoms and a good response to antibiotic treatment and those are four important criteria in diagnostic of Lyme borreliosis (Dmitrović and Popović 1993). Laboratory testing has to be validated in order to be useful in diagnostic of Lyme disease because false positive and false negative findings are possible (Jacobson et all, 1996). According to the OIE Manual, recommended methods for diagnostic of Lyme disease are ELISA test, indirect fluorescence (IFA) or immunoblot method (Western blot). Dogs disposed to *B.burgdorferi* s.l. are in 95% cases seropositive, but asymptomatic carriers of the disease.

4.1 Field research

Field research was done by field collecting of ticks with „flag" method. Chosen localities for tick collecting were in Northern part of Serbia, province of Vojvodina and localities in Fruska Gora mountain. Localities were chosen with different vegetation, from simple grass fields, fields with weeds by the channel and river with sandy soil, to forested sites of Fruska Gora mountain – urban sites, parks, abounded settlements, suburbs, green fileds and settlements

beside Danube river, forestery sites in Fruska Gora, enbankments. The conditions and habitats were optimal for tick life cycle. Also animals and humans were present in the chosen localities and sites. Localities were chosen according to the previeus history of tick bites in humans and dogs, previous research on tick infection with *B. burgdorferi* s.l. and seroprevelance in dogs for Lyme disease. Ticks were collected from the end of March to the beginning of June and then again from August until the end of October. From all the ticks collected, after identification, only *Ixodes ricinus* ticks were further analyzed. Pools were made on the basis of stage and gender and by microscopy in dark field ticks abdomen was explored and analyzed for the presence of spirochetes. When spirochetes were found, the samples of the abdomen content were inoculated into liquid BSK-H complete medium (Sigma medium complete with 6% of rabbit serum) and kept at 33^{o}C (Cisak et all, 2006; Pejchelova, 2007). Examination of ticks was done according to the work of Milutinović et all, 2004.

During a three year period, 1224 ticks were collected from different localities and 62% of them were *Ixodes ricinus*. After examination of tick abdomen, the annual percentage of infected ticks is shown in Table 1. There is an uphill trend of infected ticks during the three year period. *I. ricinus* ticks were 60-64% (764) of the total number of ticks during the research period and 22,12% of those ticks were infected with *B. burgdorferi* s.l. During the research in 2008, in one region none of the ticks were infected with *B. burgdorferi s.l.*, and in one urban region with surroundings, infected ticks were 29,2%.

Year	No of collected ticks	% I. ricinus	No and % of adult females	No and % of adult males	No and % of nymphs	**No and % of ticks infected with B.b.**
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	**1224**	**764 (62%)**	**372 (49%)**	**314 (41%)**	**78(10%)**	**169(22,12%)**

Table 1. Number of examined and infected *I.ricinus* ticks for the presence of *B.burgdorfer* s.l.i, in period 2006 - 2008.

4.2 Entomological Risk Index (ERI)

By defining ERI, the possibility of Lyme disease risk estimation for human population in a certain region can be defined. Mather et all used standard protocols to define density of tick population and their infection in different localities. Authors have also stated a positive correlation between ERI and number of Lyme disease cases in certain localities, meaning that a risk from Lyme disease for a certain region can be predicted by calculating ERI (Mather et all 1996). ERI for a certain period of research is calculated as number of collected ticks in one minute multiplied by the tick infection level. For the regions where ticks were collected, ERI was calculated in relation to a total number of collected ticks (shown in Table 2).

Region	No of collected ticks in hour	No of collected ticks in minute (NT)	Level of tick infection (TI)	ERI
Settlements Region	4	0,067	0,234	0,016
Region of FruskaGora woods	6	0,100	0,290	0,029
Urban region with surroundings	11	0,183	0,292	0,053
Mean value	**7,3**	**0,122**	**0,272**	**0,033**

Table 2. No of ticks collected in one minute (NT), level of tick infection (TI) and ERI values in the studied regions

The greatest ERI was found in urban region, because the biggest level of tick infection and total number of collected ticks was also found in the same region.

4.3 Laboratory analysis

4.3.1 Isolation and cultivation of *Borrelia burgdorferi spp*

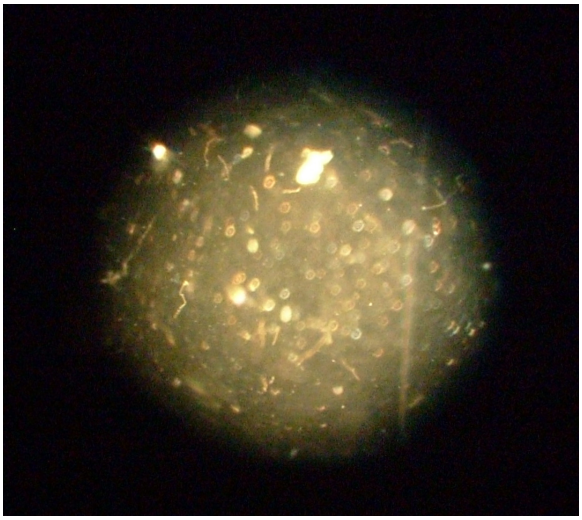
Isolation was done on selective mediom BSK-H complete medium, Sigma, according to Current Protocols in Mycrobiology (Zuckert, 2007, Pejchelova 2007). Tubes with medium and inoculated material were kept at 33-34^{o}C and after the growing of spirochetes was noticed they were recultured into a new tube, under microaerophilic conditions for growing. Typisation of spirochetes was done by a molecular method. From 26 pools cultured in total, in 4 of them (15%) the growth of spirochetes was observed and they were named after the localities they were collected at:

1. „Granicar 1“ (pool 1, 5 male adult ticks from a non settled locality);
2. „Granicar 2“ (pool 2, 5 adult male ticks from a non settled locality);
3. „244“ (pool of 5 adult female ticks from a settled locality);
4. „Novi Sad“ (pool of 5 adult ticks from the urban locality).

In first, second and third pool, growth of spirochetes was noticed after 14 days and in fourth pool after 21 days. Concentration of gained isolates of *B. burgdoerferi spp* was the following:

1. „Granicar 1“ : 32 X 10^5 *B. burgdorferi* / ml of culture;
2. „Granicar 2“ : 32 X 10^5 *B. burgdorferi* / ml of culture ;
3. „244“ : 72 X 10^5 *B. burgdorferi* / ml of culture;
4. „Novi Sad“ : 76 X 10^5 *B. burgdorferi* / ml of culture.

Detection of specific antibodies against *B. burgdorferi s.l.* was done with serologic methods CF, ELISA, Western blot and fast test. The observation for the apperiance of clinical symptoms of Lyme disease was also done. In the localities where ticks were collected, blood sampling from dogs was done, for serological survey. Dogs blood samples are deviden into three groups by their usage, region and existing clinical symptoms of Lyme disease.



Picture 5. Spirochetes of „Novi Sad“ isolate in dark field

5. Serology

Serology was done in the blood serum from dogs naturaly infected *B. burgdorferi* s.l. Samples were from:

- dogs with clinical symptoms of Lyme disease,
- dogs pets without any clinical symptoms, from the region where *B. burgdorferi* s.l. was found to be present in ticks,
- hunting dogs and military dogs without any clinical symptoms, from the region where *B. burgdorferi* s.l. was found to be present in ticks.

CF (complement fixation) method was done in microtitar plates, using produsers instructions of Virion (*B. burgdorferi* s.l. antigen). Samples were diluted until 1:10 in veronal puffer and general principles of CF method were used with 2% erythrocytes in Elsevier's solution.

Method ELISA is performed by using the kis for detection of specific antibodies against *B. burgdorferi* s.l. by Mikrogen, Germany - recomWell Borrelia canis IgG and recomWell Borrelia canis IgM. This enzyme immunotest contains recombinant antigens for the detection of IgG or IgM antibodies against *B. burgdorferi sensu stricto, B. garinii and B. afzelii*, in dog's serum or plasma samples. This is a quantitative test for detection and identification of IgG or IgM antibodies and represents a screening test based on the principles of indirect sandwich ELISA. By using recombinant proteins, the usage of protein combinations of different *B. burgdorferi* genospecies in one test is possible and by selecting specific antigens, important for a sensitive serologic diagnostic, the influence of cross reactions in the test is very low. In this ELISA test, proteins of the outer membrane OspC and specific inner part of p41 antigen (flagelin) are used and also a very specific *B. burgdorferi* s.l. antigens like p100, VisE and p18 (a bounding protein A, Osp17). Although, it is possible that if the analysis is done in the real early phase of infection when there are not enough antibodies yet, that a false negative finding appears. Therapy with antibiotics in the early stage of the disease can also reduce the amount of

antigens needed and that way influence the results of ELISA test. So, the producer recommends that if the ELISA findings are negative and clinical symptoms exist, a new sample and testing should be done after three weeks. False positive results are also possible, because antibodies persist in the organism for a long time and they can be from a previous old infection, still high enough to induce a positive finding. That is why it is recommended to confirm a positive finding by another method, like Western blot. The findings after ELISA test have o be interpreted together with having in mind clinical symptoms.

Western blott test is performed by using the kits according to the producers instructions Mikrogen for diagnostic kit recomBlot Borrelia Canis IgG. RecomBlot Borrelia canis is a qualitative test for in vitro detection and assured identification of IgG or IgM antibodies against *B. burgdorferi* s.l. in serum or plasma from dogs. This test can be used for verification of the results gained after a screening test. In recomBlot Borrelia canis test, specific *B.burgdorferi* s.l. antigens are used which are produced with recombinant cells of *E. colli*. Antigens used in the reaction are proteins of outer surface OspA and OspC, and also highly specific B. *burgdorferi* antigens p100, p39 and p18, and also p41 (flagelin). In recomBlot Borrelia canis test OspC protein from all three genospecies can be found. By immuno blot testing it is possible to distinguish a natural infection from vaccination. A negative finding still does not completely rule out the possibility of *B. burgdorferi* s.l. infection, especially in the very early phase of infection, when there are still not enough antibodies produced in the organism. Also, antibiotic treatment in the really early stage of infection can prevent forming detectible antibodies. If there are clinical symptoms for Lyme disease and a negative finding by Western blot method, the reaction should be repeated three weeks later. In the early stage of infection, IgM antibodies are predominant and reaction with OspC and p41 (IgM). In the later stage of infection, beside with OspC and p41, there is a strong reaction also with p100 and VisE. Detection of IgG antibodies against p100, p39 or p18 is enough for a positive finding.

A fast test FASTest Lyme, by Mega Cor Diagnostik, Austria is a sensitive and reliable method, based on specific imunochromatography. It is produced so that IgG and IgM antibodies can be detected in dog's blood serum or plasma. In the test there is a unique combination of specifically marked antigens, with conjugate color. For performing the test there are no special conditions needed and the result comes out in 15 minutes. In endemic regions a seropositive finding can be gained, without any clinical symptoms, so every positive result has to be confirmed by a specific ELISA or Western blot test.

Blood samples from dogs were divided into three groups:

In the first group there were 145 blood samples from working and hunting dogs that live in the regions where ticks were collected previously. All the dogs in this group were regularly vaccinated and treated against ecto and endo parasites, age from 1 to 10, or different breeds – Hungarian visla, German hunting terrier, Labrador, pullin, Rottweiler, epaniel Breton, German Sheppard and sarplaninac . These dogs were constantly exposed to ticks, every day and in 19 (13%) dogs the ticks were found while blood sampling. In 64 (44%) dogs at least one tick was found in the previous period. These dogs are used for hunting, field work or military purposes. With CF test a positive finding was gained in 22,1% of samples. In the samples from the region where infected ticks were not found, there were also no positive finding by serology, with CF and ELISA. The greatest number of dogs with positive

serology findings for specific antibodies against *B. burgdorferi s.l.* was in the region of settlements 37,9% by CF and 34,5% by ELISA test. In total, the percentage of dogs from firs group that have specific antibodies against *B. burgdorferi s.l.* is 22,1% by CF method, 22,4 % by ELISA test and 18% by Western blot method.

In the second group there were 16 blood samples from dogs with clinical symptoms interpreted as Lyme disease symptoms – lameness, difficult waking, repeated lameness in different limbs, loss of appetite, occasional elevation of body temperature 39,5-40^{o}C, weakness, with a history of tick bite in the last 2 months. In dogs from this group clinical symptoms were observed. This was done with a help of veterinary practices from the region. Six of these dogs were on a nonspecific unsuccessful treatment with antibiotics of wide range before the blood analysis. These dogs had a repetition of lameness every time the antibiotic treatment was stopped. The serology tests done in these dogs were fast test, CF, ELISA and Western blott. As a result a positive serological finding was gained in 15 dogs by CF, in 14 dogs by ELISA and Western blott and in 13 dogs by fast test. In two dogs specific antibodies against B. *burgdorferi* s.l. were not found by four serology tests. In the same 13 samples the presence of specific antibodies against *B. burgdorferi* s.l., were found with four different serology tests.

In the third group there were 486 blood samples from dogs which were brought for different analysis (piroplasmosis, leptspirosis, leishmaniosis, noninfective digestive malfunctions, urinary problems, ascites, bacterial skin infections, dirofilariosis, respiratory problems, etc), with clinical symptoms not related to Lyme disease. These dogs were kept as pets, mostly in the urban region with surrounding and some from the Fruska Gora Mountain. Dogs were from 2-14 years old. In this group were also samples from 39 dogs living in two dog shelters, all dogs were older than 5 years with an "unknown history". All of these dogs came from the region where ticks were collected previously and where ticks infected with *B. burgdorferi* s.l. were found (29%). Serology was done by CF method, and then positive samples were tested with ELISA and Western blot method. By CF method a positive finding was gained in 32,9% of samples (24,3% in dilution 1:10 and 8,6% in dilution 1:20). By ELISA test a positive finding was gained for IgM antibodies in 19,3% of samples and for IgG in 25,5% of samples. In total, positive serology finding by ELISA was gained in 29,6% of blood samples from the dogs in the third group. By Western blott positive serology finding was gained in 26,1%. In 107 samples analyzed (out of 127 with positive finding) a p100 was detected, which is characteristic for *B. afzelii*. In 55 samples protein BmpA (39) was detected, also characteristic for *B. afzelii*, and in 54 samples VisE was found, which is characteristic for different genospecies. In 22 samples protein p41 was found, characteristic for *B. burgdorferi* sensu stricto. OspA characteristic for *B. afzelii* were detected in 18 samples and OspC characteristic for different genospecies were detected in 19 samples. The highest number of samples gave positive findings for the proteins characteristic for *B. afzelii*.

A comparative display of the findings after serology test of three groups of dogs blood serums analyzed for the presence of specific antibodies against *B. burgdorferi* s.l. by four serology methods – fast test, CF, ELISA and Western blot is given in Table 3. The highest number of positive findings was gained by CF method (32%), then by ELISA (29,91%) and by Western blot (25,81%). In the total of 631 dogs blood samples without clinical symptoms of Lyme disease living in the region where seroprevalence in ticks is 22%, a seroprevalence for Lyme disease in dogs was found to be 21,4% - 32,9%, depending on the method used.

No of group	Total No of samples	No of positive samples to B.b.s.l. by fast test	No of positive samples to B.b.s.l. by CF	No of positive samples to B.b.s.l. by ELISA IgM	No of positive samples to B.b.s.l. by ELISA IgG	No of positive samples to B.b.s.l. by ELISA	No of pos. samp.to B.b.s.l. W.blott
I	145	-	32 (22,1%)	15 (10,3%)	27 (18,6%)	31* (21,4%)	26 (18%)
II	16	13 (81,25%)	15 (93,75%)	3 (18,7%)	11 (68,7%)	14* (87,5%)	14 (87,5%)
III	486	-	160 (32,9%)	94 (19,3%)	124 (25,5%)	144* (29,6%)	127 (26,1%)
Total	647	13	207 (32%)	112 (17,31%)	162 (25,04%)	189* (29,91%)	167 (25,81%)

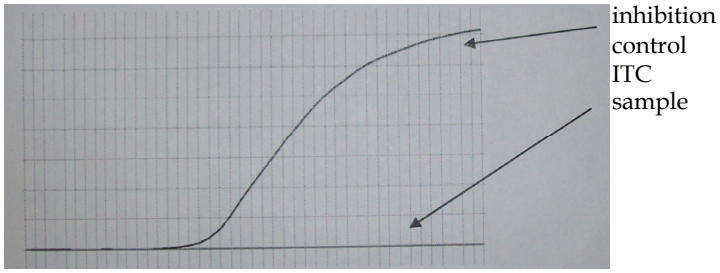
Not a simple adding of positive IgG and IgM, because of overlapping in some samples of positive finding to IgG and IgM ELISA (74 samples in total)

Table 3. Results of analysed blood samples from dogs, for the presence of specific antibodies against *B. burgdorferi* s.l.with four serology tests

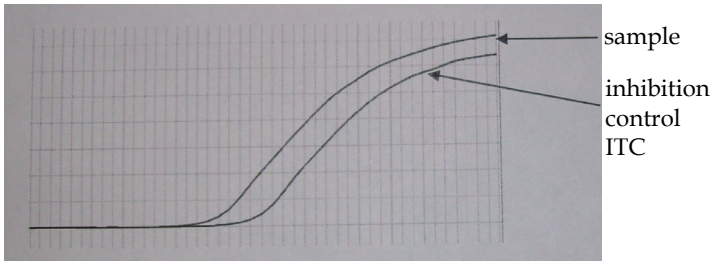
6. Molecular analysis

PCR (Polymerasa Chain Reaction) and Real Time-PCR were used for identification of *B. burgdorferi* s.l. isolated from ticks and typisation of genospecies from isolates of *B. burgdorfer*i s.l. from ticks. Isolation of total DNA was done with QIAamp® DNA Mini kit, with „spin-column" procedure of „QIAGEN", Germany. Identification of *B. burgdorferi* was done by real time PCR method with a diagnostic kit PCRFast® *Borrelia burgdorferi* Realtime (SYBR®Green) and / or gel detection, Germany. In case of realtime (SYBR®Green) positive findings, a verification has to be done by analysis od dissosiation curve and evaluation of exponential curve of amplification. For that purpose the picks of dissosiation curve belonging to the sample are compared with the ones belonging to the inhibition control ITC. In estimation of the results exponential curve of amplificatin and dissosiation curve are beeing analysed. Exponential curve of amplification of the sample and inhibition control ITC are beeing compared and they should have similar values. In positive samples dissosiation curve and curve of inhibition control should be at the same level. In negative samples there is no dissociation curve or it is different from the dissociation curve of inhibiton control ITC (Picture 6).

Dissociation curves from samples of isolated cultures from field ticks had similar values with dissociation curve for each positive control, inhibition control ITC, meaning that the samples were positive for the presence of *B. burgdorferi* s.l. DNA. *B. burgdorferi* s.l. is identified in the cultures of spirochetes isolated from field ticks. For the isolate „Novi Sad" pick temperature of dissociation curve was 79,0^{o}C, and pick temperature of ITC for the same sample was 79,4^{o}C.

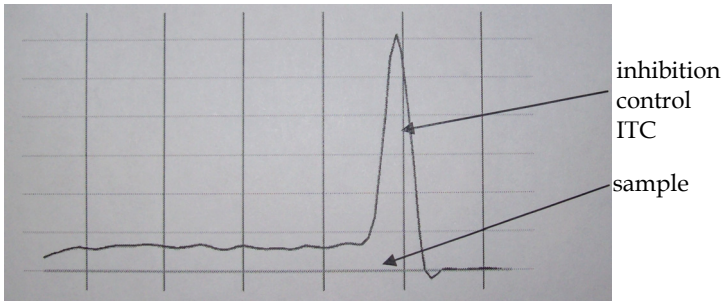


Negative sample

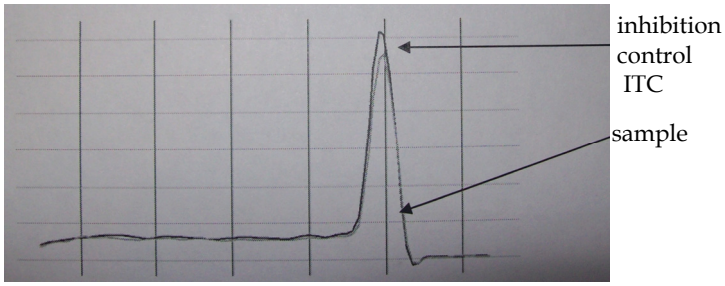


Positive sample

Picture 6. Evaluation of dissosiation curve



Negative sample

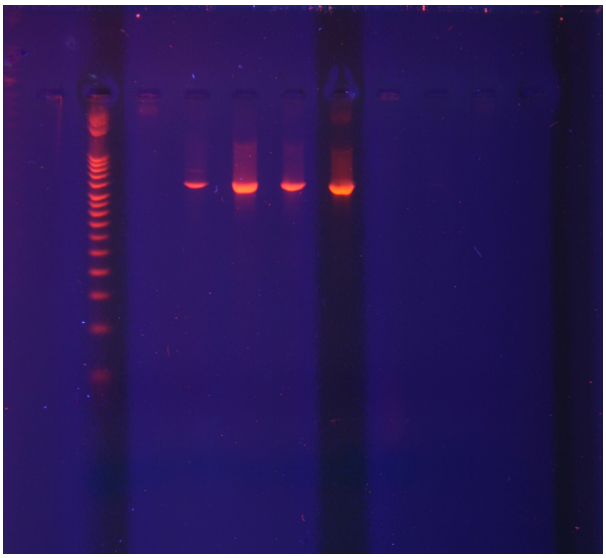


Positive sample

Picture 7. Evaluation of exponential curve of amplification

Typisation of *B. burgdorferi* s.l. isolates from ticks was done by PCR technique and the results were the following:

1. „Granicar 1" : *Borrelia afzelii* (specific fragment 591bp);
2. „Granicar 2" : *Borrelia afzelii* (specific fragment 591bp);
3. „244" : *B. burgdorferi sensu stricto* (specific fragment 575bp);
4. „Novi Sad" : *Borrelia afzelii* (specific fragment 591bp).



Picture 8. Typisation of *B. burgdorferi* s.l.genospecies of isolates by PCR technique: species specific PCR for *B. burgdorferi* s.l. isolates; M: DNA size marker specific fragment 50bp; 1:Pool „244"isolate amplified with specific primers for *B. burgdorferi sensu stricto*; 2-4: Pools „Granicar 1 and 2"andi „Novi Sad" isolates amplified with specific primers for *B. afzelii*

6.1 Statistics – Kappa test

Data gathered during the research was analysed by Kappa statistic test (Valčić 1998). This test is useful for the analysis of methods which as a result have „posotove-negative" values, instead of numeric values. Statistical analysis with Kappa method is used for comparing of compatibility of findings among two tests or methods. Kappa value 1 is for a complete and ideal comatibility and 0 is for a accidental compatibility of tests. All the other values can be in between. Compatibility of methods Western blot and ELISA for serological findings in three groups of dogs is 0,915, meaning that the compatibility of these two methods is ideal. Compatibility of fast test and ELISA (or Western blot, because the values were the same for those two tests) for serological findings in second group of dogs after natural infection, is also high, its 1, maening that the compatibility of methods is ideal or complete. CF method does not have complete compability compared to ELISA or Western blott. This means that ELISA or Western blott can be eaqualy used in diagnostics of Lyme disease.

6.2 Resume of Lyme disease in ticks and dogs in Serbia

In Serbia, research on ticks as vectors of infectious agents started over 60 years ago. Lyme disease was discovered in 1987, and more intensive research on ticks, their ecology, seasonality... Over the years there have been some changes becauseof the changes and irregularities in climate and metrology factors. Reaserch done in the last several years in Serbia have prooved infection in I. ricinus ticsk with *B. burgdorferi* between 20 – 30% (Milutinović et all, 2004; Jurišić, 2008; Cekanac et all, 2009), and in some parts even over 40% (Milutinović et all, 2008). During this three year period of research in chosen localities an average infection of ticks with *B. burgdorferi* s.l. is 22,12%. The highest percentage of infected ticks was found to be in urban region with surrounding (29,2%) and in one locality (in Banat region), there were no infected ticks found at all.

The average value of ERI found in this research was 0,033. The highest risk from Lyme disease was found to be in urbane region with surroundings (0,053), where also was highest rate of ticks infected with *B. burgdorferi* s.l. In literature it is mentioned that the value of ERI is in correlation with the number of human cases with Lyme disease. Calculated value of ERI in a region can be useful information in estimation of the risk from spreading of Lyme disease in a certain region and also for the prediction of appearance of Lyme disease in humans (Mather et all, 1996).

From the ticks collected in the region, four autochthonous strains of *B. burgdorferi* s.l. were isolated and three of those strains belonged to genospecies *B. afzelii,* and one belonged to genospecies *B. burgdorferi sensu stricto*. Several authors worked on isolation of Borrelia from ticks and their identification and typisation to the genospecies level with method based on PCR technology (Cerar et al 2009, Lindblom et all 2009, Wilhelmsson et all 2009). Results on the outspread of *B. burgdorferi* genospecies in vectors of Lyme disease show a dominant presence of *B. afzelii*, compared to a less frequent occurrence of *B. burgdorferi sensu stricto* in ticks. Additionally, the findings after analysis done with Western blot highlight the same result: from the total of 127 dogs with a seropositive serology finding for *B. burgdorferi*, in 107 dogs, the presence of specific antibodies to protein antigens characteristic for *B. afzelii* were found. In previous research done in the region of Serbia (different localities), the domination of *B. afzelii* was also found, compared to *B. burgdorferi sensu stricto* and *B. garinii* (Cekanac et all, 2009).

Clinical symptoms were found in a certain number of dogs. Since in most of the dogs specific antibodies against antigens of genospecies B. *afzelii* were confirmed, it can be stated that in Serbia region where the research was done, cases of Lyme disease in dogs is mostly caused by *B. afzelii* with general clinical symptoms of difficult moving, lameness, lethargy, irregular elevation of body temperature up to 40^{o}C, loss of appetite and weakness. Two groups of dogs were analyzed which did not have clinical symptoms that could be related to Lyme disease and a prevalence was found for *B. burgdorferi* s.l. In group of dogs used as working, hunting and military dogs the prevalence was in the range from 21 – 37%, depending on the laboratory method used and in the group of dogs which were kept as pets the prevalence for *B. burgdorferi* was in the range from 26 – 33%. The prevalence found in one group of dogs was not much different than in the other, even though one group is constantly exposed to the tick influence, so the risk from occurrence of the disease is pretty much the same no matter if dogs are being used for hunting or as pets, as long as they are protected by antiectoparasitic products.

A significant difference in the percentage of positive findings during the research was gained, related to the method used for diagnostic, in dogs with Lyme disease. Author Jovičić in her research concludes that ELISA test should be used at the beginning of diagnostic process in humans suspicious for Lyme borreliosis and Western blott is to be used as conformation method (Jovičić, 2001). The findings from this research pretty much backup this statement. In this research, in dogs with clinical symptoms of Lyme disease , the greatest number of positive serology findings was gained by CF method (93,75% of positive samples). But far more balanced and unified results were found by ELISA (87,5% positive samples), Western blot (87,5% positive samples) and fast test (81,25% positive samples). CF method in diagnostic of Lyme disease can only be used as „screening" method for research purposes and in everyday routine this method is nor useful. Fast tests can be used for first glance diagnostic, for fast and orientational result. If there is a dog with clinical symptoms that could indicate to Lyme disease, fast tests should always be used as first method. Every positive finding should be confirmed by another method like ELISA or Western blot. If fast test gives a negative result and there is still a suspicion of Lyme disease, analysis should be repeated with a more sensitive method (ELISA or Western blot) in a certain time interval, for a more complete and confident diagnosis. Several authors recognize ELISA as the most convenient method for monitoring and clinical check up of Lyme disease in dog population, because it is a highly sensitive test with objectivity in resulting (Magnarelli et all, 1988, Goosens et all, 2000). Western blott is often described by the authors as conformation method and proteins of molecular weight in the range from 66–73 kDa (in this research VIsE from 66kDa was used) are considered to be dominant immunogenes present in every pathogen genospecies of *B. burgdorferi sensu lato* (Luft et all, 1991). In this research by Western blott method in the majority of positive samples for Lyme disease, antibodies for protein antigens of genospecies *B. afzelii* were detected (84%).

In dogs that did not have clinical symptoms indicating Lyme disease an "accidental" positive finding should be interpreted very carefully, having in mind all the relevant data for making a definite diagnosis. There are fast tests that can diagnose few diseases at once like fast test for erlichiosis, dirofilariosis and borreliosis. Usage of these tests can mislead in diagnosis if initially there is a suspicious for another disease and the test gives a positive result for Lyme disease. Serology testing for Lyme disease does not have any value in predicting the condition in limbs or joints (Levy and Magnarelli, 1992.). For exact diagnosis it always has to be asked if dog has spent some time in a region which is endemic for Lyme borreliosis. After infection, 4-6 weeks is needed for immunoresponse and before this period is over, a negative serology result can be found even if a dog is infected. Diagnosis in Lyme disease has to be done based on epizootiological anamnesis, clinical check up and laboratory analysis.

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