

STUDY ON CLINICAL AND LABORATORY DIAGNOSTIC OF LYME DISEASE IN DOGS AFTER EXPERIMENTAL INFECTION

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*Experimental infection was done on 13 dogs, with *B. burgdorferi* s.l., in the epizootiological area where Lyme disease in dogs and humans is present. Prior to the experimental infection, dogs in the experiment had no contact with *B. burgdorferi*, and they were kept in isolation. Serological methods used in the study were complement fixation and ELISA test. Biochemical blood analysis was done, also. The experimental infection of dogs was done with a referent ATCC *B. burgdorferi* s.l. culture, and with the isolates of *B. burgdorferi* s.l. previously gained from *Ixodes ricinus* ticks collected on selected locations of the observed region in the northern part of Serbia (Vojvodina province).*

After the experimental infection, clinical symptoms were not seen in dogs and positive serological results were found in 70% of experimentally infected dogs.

*Immunodiagnostic criteria for the diagnosis of Lyme disease in dogs are established. In dogs without clinical symptoms for Lyme disease, when clarifying the laboratory results, one must have in mind the epizootiological situation of the region and also the possibility of former contact of the dog with *B. burgdorferi* s.l. For epizootiological surveys, CF can be used as an approximate screening method, with obligatory conformation with ELISA in the case of positive findings.*

Key words: diagnostic methods, dogs, experimental infection, Lyme borreliosis

INTRODUCTION

Lyme disease is one of the diseases discovered within the last three to four decades. It is a systemic, infectious, vector borne and zoonotic disease, caused by a spirochete *Borrelia burgdorferi* s.l. Ticks transmitting Lyme disease in Europe are of the *Ixodes ricinus* species (Burgdorfer, 1989). There are 13 strains of *Borrelia burgdorferi* s.l. known so far and only three that are pathogen for dogs and humans: *B. burgdorferi sensu stricto*, *B. afzelii* and *B. garinii*. After natural

infection in dogs, with *Borrelia burgdorferi* s.l. clinical symptoms can be found in 5% of infected dogs. In most cases the first symptoms are anorexy, weakness, lymphadenopathy, increase of body temperature. Later on, 2-5 months after a tick bite intermittent lameness can be found, because of the mono or oligo arthritis, which can last from several days to few weeks. According to the published data (Appel *et al.*, 1993; Straubinger *et al.*, 1997; Chang *et al.*, 2001), after experimental infection in dogs, clinical symptoms are not an obligatory finding. Sometimes symptoms like raised body temperature and intermittent lameness can be the only ones found. Serological findings were positive and the isolation of *Borrelia burgdorferi* s.l. can be done from skin, axilar lymph nodes and rarely from the synovial fluid. In dogs with no clinical symptoms after the experimental infection, positive serological findings for *Borrelia burgdorferi* s.l. were gained. Some researchers found even 75% of infected dogs with clinical arthritis after experimental infection, where mono or oligo arthritis was found 2-5 months after exposure to the infection (Appel, 1993; Straubinger, 1997). Burgess in his experiment injected s/c a culture of borrelias isolated from a mouse and obtained a serological response within 8-28 days after inoculation with no clinical symptoms (Burgess, 1986). Some authors found only a raise of body temperature and arthritis after a while which caused lameness (Appel *et al.*, 1993; Chang *et al.*, 2001; Straubinger *et al.*, 2000).

Due to the non-characteristic clinical symptoms, diagnostics of Lyme disease is complex. Usually in routine work clinical findings and history are insufficient for a definite diagnosis. Today, diagnosis of Lyme disease in dogs and humans is based on the detection of specific antibodies against *Borrelia burgdorferi* s.l, isolation and cultivation of the causative agent from tissues, and molecular diagnostic by PCR.

Since the diagnostic of Lyme disease is complex, it is usually based on epidemiology data, clinical symptoms, laboratory tests and reaction to antibiotic treatment (Skotarczak, 2007). The recommended methods for the diagnosis of Lyme disease according to the OIE Manual are ELISA, IFA and immunoblot methods. The problem with serological methods is the possibility of a cross reaction with other spirochetes if the method is not specific enough or the persistence of antibodies is long after the infection. Appel *et al.* (1993) showed that there is a significant difference in serological response in dogs which were inoculated with *Borrelia burgdorferi* s.l. cultivated *in vitro* compared to the one in dogs experimentally infected via ticks.

Research in the world (Joppert *et al.*, 2001) and Europe (Pejchalova *et al.*, 2007; Smetanova *et al.*, 2007; Moran Cadenas, 2007) show different data on the existence of pathogen genospecies of *Borrelia burgdorferi* s.l. in ticks and clinical manifestations of the disease in dogs and humans. Data on the prevalence of Lyme disease in dogs and humans are also published more than once for different regions (Goossens *et al.*, 2001; Renaud *et al.*, 2004; Ružić-Sabljić *et al.*, 2005). In Serbia, during the last five years, it has been found that 25-30% of ticks are infected with *Borrelia burgdorferi* s.l. depending on the region (Jurišić, 2008; Milutinović *et al.*, 2008; Cekanac *et al.*, 2009; Savić *et al.*, 2010). There are also cases of Lyme disease in dogs and humans with clinical signs and positive

serological findings. Seroprevalence for Lyme borreliosis in dogs has been previously determined for the northern part of Serbia as 25.81% and also the seroprevalence of ticks in the same region as 22.12%. Genotypisation of *Borrelia burgdorferi* s.l. was done and *B. burgdorferi sensu stricto* and *B. afzelli* were found in this region (Savić et al., 2010).

MATERIALS AND METHODS

The experimental infection was done on mixed breed dogs of different age, born and raised under controlled conditions, with no chance of getting into contact with ticks. Dogs were kept in boxes with a concrete floor and wooden houses, divided by sex and age. All the procedures done within the experiment were carried out in a humane way, according to valid standards and laws. Dogs were observed daily, identified with names, but in the results each dog was presented with a number for easier manipulation with the data. In total, 13 dogs were used for the experiment: six for the experimental infection with ATCC referent strain *Borrelia burgdorferi* s.l., four for the experimental infection with previously isolated strains of *Borrelia burgdorferi* s.l. from ticks, and three dogs were for control. There were 9 adult dogs (3-4 years) and 4 dogs were less than one year old at the beginning of the experiment. There were seven females and six male dogs in the experiment. Control dogs were all the time in cohabitation with experimental dogs (Table 1). The maintenance of isolates was done according to a determined protocol (Zuckert, 2007).

Table 1. Experimental infection of dogs with a referent ATCC strain and isolated autochthonous isolate of *B. burgdorferi* s.l.

	Exp.infection with ATCC strain	Exp.infection with "Granicar" strain	Exp.infection with "Novi Sad" strain	Control group	Total
Total No of dogs in group	6	2	2	3	13
No of females	4	1	1	1	7
No of males	2	1	1	2	6
Adults	3	2	2	2	9
Dogs less than one year old	3	0	0	1	4

Experimental infection of 6 dogs by i/c application of ATCC strain was done with a referent ATCC strain of B. burgdorferi s.l.: ATCC-35210 B. burgdorferi (Borrelia burgdorferi ATCC® 35210), serial No 7688842. The culture was kept in BSK-H medium (Sigma). The application was done on the right side, in the shaved 5 X 5 cm neck and prescapular region, with 300 µL B. burgdorferi s.l., concentration 74 X 10⁵ bacteria/mL culture, in BSK-H media. The application was

supposed to "imitate" a tick bite. Before application a "zero" sample of blood was taken from dogs.

Dogs were observed daily for 105 days (15 weeks) – body temperature, place of application, surrounding lymph nodes, changes in appetite, behavior, vomiting, apathy and movement of animals. During the first 3 weeks the blood samples were taken twice a week. For the next 2 weeks sampling was done once a week and after that every two weeks until day 105 after experimental infection. After the "0" sample, blood sampling from 1. - 8. was done on the 4th, 7th, 11th, 14th, 18th, 21st, 28th and 35th day after the experimental infection. Sampling from 9. - 13. were taken every 14 days, and the last sampling was done 105 days after the experimental infection.

Experimental infection of dogs by i/c application of previously isolated B. burgdorferi s.l. from field ticks was done on the left side of the body, with two isolated strains, named by the locations that the ticks were collected from:

- "Graničar" concentration 32×10^5 *B. burgdorferi*/ml – two dogs, and
- "Novi Sad" concentration 76×10^5 *B. burgdorferi*-ml two dogs.

Dogs were also observed daily for 49 days (isolate "Novi Sad") and 63 days (isolate "Granicar") the same as described. Blood samples were taken from *v. caephalica*. From blood samples serological analysis was done by CF and ELISA method and also biochemical analysis. During the first 3 weeks samples were taken twice a week and then once a week. After "0" sample, blood sampling from 1. - 6. was done on the 4th, 7th, 11th, 14th, 18th and 21st day after the experimental infection and sampling from 7. - 10. (isolate "Novi Sad") or to 12. (isolate "Granicar") were done every 7 days.

Serological methods used for the analysis of blood samples were CF (with *B. burgdorferi s.l.* antigen, from Virion) and ELISA IgG and IgM test (recomWell *Borrelia canis*, Mikrogen, Germany).

Biochemical analysis of blood was done only in the samples that were previously confirmed as positive for Lyme borreliosis by serology (CF and ELISA). The parameters measured were: total protein, albumines, globulines, AST, ALT, urea, cholesterol, triglycerides, calcium and phosphorus with the aim to see if any of the parameters changed significantly.

Statistical analysis of the data was done by Kappa test for the checkup of compatibility of the results.

RESULTS AND DISCUSSION

After the experimental infection of 6 dogs with referent ATCC strain *B. burgdorferi s.l.* none of the infected dogs had any changes in behavior nor clinical symptoms. The changes were not seen at the place of the application or any other place on the skin. After the experimental infection of 4 dogs with autochthonous isolates "Novi Sad" and "Graničar", also none of the infected dogs had any changes in behavior nor clinical symptoms. The changes were not seen at the place of the application or any other place on the skin. In control dogs there were also no clinical changes.

Serological findings after experimental infection of dogs with *B. burgdorferi* s.l are shown in Table 3. Serological findings by CF and ELISA for control dogs were negative during the whole experiment.

CF results:

After the experimental infection of dogs with referent ATCC strain of *B. burgdorferi* (7688842), serological findings with CF were the following: two dogs showed no seroconversion at all; from the remaining 4 dogs with seroconversion, the earliest positive finding was detected on the 14th day after the infection. The highest antibody titer was 1:20, existing from 18th day after infection. Antibody titer could be detected up to 49 days after the experimental infection.

After the experimental infection of dogs with isolates of *B. burgdorferi* "Novi Sad" and "Graničar" with CF method serological findings were the following: one dog showed no seroconversion at all; in the remaining 3 dogs with seroconversion the earliest positive finding was detected on the 7th day after the infection, the highest antibody titer was 1:20 existing from 11th – 14th day after infection. Antibody titer could be detected the latest until 56 days after the experimental infection. All the findings with CF method after experimental infection of dogs with the referent strain and isolates of *B. burgdorferi* s.l. are shown in Table 2.

Table 2. Results after the experimental infection of dogs with referent ATCC strain and "Novi Sad" / "Graničar" isolates of *B. burgdorferi* by CF method

No of dogs with Titer	No sampling/day	0	1/4 th	2/7 th	3/11 th	4/14 th	5/18 th	6/21 st	7/28 th	8/35 th	9/49 th	10/63 rd	11/77 th	12/91 st	13/105 th
	Strain														
1:10	ATCC	0	0	0	0	2	3	3	3	3	2	0	0	0	0
1:20	ATCC	0	0	0	0	0	1	2	2	2	0	0	0	0	0
No of dogs with Titer	No sampling/day	0	1/4 th	2/7 th	3/11 th	4/14 th	5/18 th	6/21 st	7/28 th	8/35 th	9/42 th	10/49 rd	11/56 th	12/63 st	-
	Strain														
1:10	"N. Sad"	0	0	0	0	1	1	1	1	1	0	0	0	0	-
	"Granicar"	0	0	1	1	1	2	2	2	1	1	1	1	0	-
1:20	"N. Sad"	0	0	0	0	0	0	0	0	0	0	0	0	0	-
	"Granicar"	0	0	0	1	1	0	0	0	0	0	0	0	0	-

ELISA results:

After the experimental infection of dogs with referent ATCC strain of *B. burgdorferi* (7688842), serological findings with ELISA test were the following: 3 dogs showed no seroconversion at all. In the 3 dogs with seroconversion, the

earliest positive finding of IgM was detected on the 7th day after the infection. Positive finding of IgG was detected 11 days after the experimental infection. Antibody titer for IgG could be detected until 28th day after the experimental infection. After the 35th day antibodies against *B. burgdorferi* s.l could not be detected any more by ELISA IgM or IgG method.

After the experimental infection of dogs with isolates of *B. burgdorferi* "Novi Sad" and "Graničar", serological findings with IgM and IgG ELISA test were as follows: one dog showed no seroconversion at all during the experiment; in the rest 3 dogs the earliest positive finding of IgG was detected on the 7th day after infection. Antibody titer could be detected until 35 days after the experimental infection in two dogs, and in one dog even until the 56 day. After 56 days antibodies against *B. burgdorferi* s.l could not be detected any more by ELISA IgM or IgG method. The findings with ELISA IgG method after the experimental infection of dogs with referent strain and isolates of *B. burgdorferi* s.l. are shown in Table 3.

Table 3. Results after the experimental infection of dogs with referent ATCC strain and "Novi Sad" / "Graniča" isolates of *B. burgdorferi* by ELISA IgG test

	No sampling/ day	0	1/ 4 th	2/ 7 th	3/ 11 th	4/ 14 th	5/ 18 th	6/ 21 st	7/ 28 th	8/ 35 th	9/ 49 th	10/ 63 rd	11/ 77 th	12/ 91 st	13/ 105 th
	Strain														
No of dogs with IgG Titer	ATCC	0	0	0	1	3	3	3	2	0	0	0	0	0	0
	No sampling/ day	0	1/ 4 th	2/ 7 th	3/ 11 th	4/ 14 th	5/ 18 th	6/ 21 st	7/ 28 th	8/ 35 th	9/ 42 th	10/ 49 rd	11/ 56 th	12/ 63 st	-
	Strain														
	"N. Sad"	0	0	0	1	1	1	1	1	1	0	0	0	0	-
	"Granicar"	0	0	1	2	2	2	2	2	1	1	1	1	0	-

During the whole period of seroconversion, clinical symptoms were not seen. This supports the opinion that in Lyme disease cases, in dogs the causative agent can be present in the organism, with no clinical symptoms (Savić-Jevdjenić, 2008). This may cause a debate during the diagnostic process and requires a decision if therapy is needed or not, especially in cases found positive as a side result (for expl in multiple fast tests).

The results after the experimental infection of dogs with referent strain and tick isolates of *B.burgdorferi* s.l. showed no clinical symptoms characteristic for Lyme disease. Seroconversion was found in 60% of dogs, with ELISA test and in 70% of dogs with CF test. The earliest antibodies were discovered seven days after the experimental infection and the longest they could be found was 56 days after the experimental infection.

Biochemical blood test results:

Biochemical blood test analysis was done in 10 samples of dogs after experimental infection, at the time when serological finding showed positive results. Biochemical parameters in dogs after experimental infection with values outside the physiological interval were not detected. Some parameters did fall out of the physiological interval, but those were singular cases and a pattern for the elevation or decrection was not found.

Statistical results:

Results of Kappa analysis as a statistical tool are ment to show the statistical compatibility for different parameters, in this case the compatibility of positive serological findings in ELISA and CFT was 0.782 what is almost ideal compatibility.

Lyme borreliosis is a disease without characteristic clinical symptoms in dogs. Several authors have tried to induce clinical symptoms during experimental infection with *B. burgdorferi* s.l. by various ways of application (Burgess, 1986; Appel, 1993; Levine, 1995; Straubinger, 1997;).

In this study experimental infection with *B. burgdorferi* s.l. in dogs was done with referent ATCC strain of *B. burgdorferi* s.l. and autochthonous isolates previously isolated from ticks, named "Graničar" and "Novi Sad". With the analysis of blood samples after the experimental infection with CF and ELISA method, yielded findings which matched almost ideally. The earlier appearance and longer existence of serologic response was detected after the infection with autochthonous isolates of *B. burgdorferi* s.l. compared to the infection with referent strain of *B. burgdorferi* s.l. Pathogenicity of referent ATCC strain was reduced due to the subculturing in media for *B. burgdorferi* s.l. Absence of clinical symptoms was not related to one or the other isolate used for experimental infection, since in none of the dogs in the experiment clinical symptoms were found.

Laboratory isolates of *B. burgdorferi* s.l. reference strain, or isolated ones from field conditions are subcultured for a certain number of passages in the media. Laboratory isolates loose their virulence after a multiple sub-culturing in BSK-H media compared to the *B. burgdorferi* s.l. found in ticks after natural infection. The media have a certain influence to the infectiousness and pathogenicity (Wang *et al.*, 2004).

After biochemical analysis of blood samples from dogs with positive serologic finding for Lyme disease, six parameters in total were found to be changed: albumin, globulin, cholesterol, triglycerides, calcium and phosphorus. However there were no regularities in the changed parameters and no pattern (raised or reduced parameter), so these changes had no statistical, or diagnostic value.

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ISTRAŽIVANJE KLINIČKE I LABORATORIJSKE DIJAGNOSTIKE LAJMSKE BOLESTI KOD PASA NAKON EKSPERIMENTALNE INFEKCIJE

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SADRŽAJ

Eksperimentalna infekcija je izvršena sa *B. burgdorferi s.l.*, na 13 pasa u epizootičkom području gde se lajmska bolest javlja i kod ljudi i pasa. Psi korišćeni tokom eksperimenta nisu imali nikakav prethodni kontakt sa *B. burgdorferi s.l.* i držani su u izolaciji. Upotrebljavani su bili sledeći serološki metodi: reakcija vezivanja komplementa i ELISA test, a takođe je rađena i biohemijska analiza uzoraka krvi. Eksperimentalna infekcija je izvedena referentnim sojem kulture ATCC *B. burgdorferi s.l.* kulturom izolata *B. burgdorferi s.l.* koji su prethodno dobijeni iz krpelja *Ixodes ricinus* sakupljenih sa određenih lokacija posmatranog regiona u severnom delu Srbije (Vojvodina). Nakon eksperimentalne infekcije nije bilo kliničkih simptoma kod pasa, a pozitivan serološki nalaz je dobijen kod 70% eksperimentalno inficiranih jedinki.

Utvrđeni su imunodijagnostički kriterijumi za uspostavljanje dijagnoze lajmske bolesti kod pasa. Tokom tumačenja nalaza kod pasa sa kliničkim simptomima lajmske bolesti mora se imati u vidu epizootička situacija u regionu, kao i mogućnost ranijeg kontakta pasa sa *B. burgdorferi s.l.* Tokom epizootičkih istraživanja, reakcija vezivanja komplementa može da se koristi kao "screening metod, uz obaveznu potvrdu dijagnoze ELISA testom, kod pozitivnih jedinki.

