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# TESTING OF THE BIOCAN® B INJ. AD US. VET. VACCINE AND DEVELOPMENT OF THE NEW RECOMBINANT VACCINE AGAINST CANINE BORRELIOSIS

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Verification of the efficacy of Biocan® B inj. ad us. vet. (Bioveta, a.s.) was done by challenge testing. Ticks collected in the nature were used as natural vectors of the infection. Six beagles and two control ones were used in the test. Formation of outer surface protein A specific antibodies (OspA antibodies) and borrelia specific immonoglobulins (IgG) was measured by Western blot and EIA in the sera samples. The tissue samples were used for detection of borreliae by cultivation method and dark field microscopy (DFM). Formation of IgG antibodies and OspA antibodies after vaccination was observed. The maximum titer level of antibodies was reached between 21. and 49. day after vaccination and then slowly decreased. Presence of borreliae was detected only in skin biopsies of non-vaccinated dogs. The post mortem tissue samples showed presence of borreliae in all of the samples of the non-vaccinated dogs. The tissues of the vaccinated dogs were not infected with borreliae, except for two samples of dog with low titer levels of OspA antibodies.

The development of the new vaccine is based on preparation of recombinant outer surface proteins (e.g. rOspA and rOspC) of *B. afzelii*, *B. burgdorferi* and *B. garinii* origin. Chosen recombinant proteins were successfully expressed in *E. coli*. The obtained purified proteins are currently being tested on laboratory BALB/c mice. Formation of specific antibodies against some recombinant proteins has been confirmed.

These proteins are suitable candidates for preparation of a vaccine prototype and they will be subsequently used in challenge tests.

## INTRODUCTION

*Borrelia burgdorferi* sensu lato is a causative agent of chronic multi-system infectious disease, Lyme borreliosis (LB) in humans, who are dead-end hosts. Domestic animals in particular can become victims and may show similar manifestations attacking skin, joints, heart and nervous system that occur in humans.[1,2] This vector character disease is mostly transmitted by infected ticks of the genus *Ixodes*[3]. The infection is maintained in the nature by rodents, deer and birds. During the life cycle this pathogen undergoes antigen variation, an effective surface structures modification, which enables escape from the host immune system.[4,5,6] Owing to the high pathogenity and problems with autoimmune reactions in the case of whole-cell human vaccine, the development of a new vaccine using recombinant proteins is hoped to bring improvements in protective immunization for animals as well as for humans.[7,8,9] Outer surface protein A (OspA) is the major protein expressed by *B. burgdorferi* in unfed ticks, whereas outer surface protein (OspC) is up-regulated early infection in mammals.[10,11] Lyme disease patients as well as naturally or experimentally infected mice mainly produce antibodies specific for OspC rather than OspA.[12] OspA is a protective antigen, which is unusual. Its protective activity is based on formation of OspA antibodies, which eliminate the OspA-expressing spirochetes present in the tick mid-gut.[13,14] OspA vaccine has to induce sufficient amounts of OspA antibodies to prevent transmission of the pathogen.

Outer surface protein C (OspC) is also promising candidate for vaccine against LB. Some studies showed that the OspC vaccinated host could be protected from *B. burgdorferi*.[15] OspC antibodies are efficient after *B. burgdorferi* enters the host despite the OspA antibodies. The new vaccine composed of combination of OspA and OspC antigens could have dual protective effect and OspC antibodies ensure the protective activity of vaccine in that case, when the OspA antibodies are decreased.[16]

The aim of this study was to verify the protective activity of classical veterinary vaccine Biocan® B inj. ad us. vet. (Bioveta, a. s.) by the challenge test and the application of challenge test on dogs, which are suitable models for human preclinical studies and to refer about current studies of veterinary vaccine improvement, which could predate the final human vaccine.

## MATERIAL AND METHODS

Six dogs (beagles) at the age of 3-4 months were vaccinated with one dose (3 dogs s.c., 3 dogs i.m.) and boosted in one month with second dose of the Biocan® B inj. ad us. vet. vaccine. Each vaccination dose contained $10^8$ cells of inactive *Borrelia garinii* and $10^8$ cells of inactive *Borrelia afzelii* with Aluminium hydroxide as an adjuvants. Two control dogs (beagles) at the age of one month were not vaccinated. The challenge experiment was started 101 days after vaccination. All of the experimental dogs were treated with naturally infected ticks. Two months after the challenge the dogs were sacrificed.

During the challenge experiment, clinical symptoms were observed and recorded. Biopsy samples were collected. The blood samples and skin biopsies were taken for serological and cultivation examination followed by dark field microscopy (DFM). Tissue samples: skin biopsies during the infection and skin samples, lymph nodes, synovial membranes from right and left side of body were taken for post-mortal examination.

Ticks and determination of infectivity was provided by the Institute of Parasitology, Academy of Sciences, České Budějovice, Czech Republic by polymerase chain reaction (PCR) method, based on amplification of gene for outer surface protein A.[17] Ticks were applied to obtain real infection. Fixation chamber was filled with 23 ticks on each side of the dog, 46 ticks were applied per each dog, (Fig. 1). The chambers were removed in 5 days, to check the attachments of the ticks (Fig. 2).

Method of re-isolation of spirochaetes from the biopsy samples was used to detect penetration of spirochaetes into the tissues. Spirochaetes were reisolated in BSK-H complete medium (Sigma, B-8291) enriched with Gelatine (Merck, 1.04070), L-glutamine (Sigma, G-7513) and Antibiotic mixture for borrelia (Sigma, A-1956). Contamination, medium color change and spirochaetes determination by dark field microscopy (DFM) were recorded.

The blood samples were examined for OspA antibodies by using Western blot diagnostic kit (RecomBlot Borrelia IgG, MIKROGEN) and for borrelia specific IgG and IgM antibodies by using EIA diagnostic kit (EIA Borrelia IgG/IgM, TEST-LINE s.r.o., Clinical Diagnostics). ELISA KELA IgG antibodies measurement was kindly provided by Straubinger.

PCR detection of *Borrelia burgdorferi* sensu lato in the post mortem tissue samples of skin, synovial membranes, muscles and lymph nodes was performed by a service company Genex s.r.o..

Recombinant proteins purified by the method of affinity chromatography[18, 19] rOspA of *B. afzelii*, *B. burgdorferii* origin (rOspA/BA, rOspA/BB) and rOspC of *B. garinii* origin (rOspC/BG) were tested for immunogenity on the mouse model.[20] Forty inbred BALB/c mice (AnLabLtd.) were divided into four groups I, II, III and IV, ten mice per group. Each groups was actively immunized subcutaneously and boosted in 14 days with proper recombinant protein, 6 μg of rOspC/BG (group I), 6 μg of rOspA/BB (group II), 8 μg rOspA/BA (groupIII), using Aluminium hydroxide as an adjuvants, per each mousse. Group IV represented non-immunized control. Sera samples were collected 14 days after booster and examined by method of Western blot with anti-mouse-IgG (Fc specific)-AP conjugate (Sigma, A-1418).[21]

## RESULTS AND DISCUSSION

The challenge test was proposed to confirm the protective activity of the Biocan® B vaccine against borreliosis 3 months after vaccination. The application of ticks was selected as the closest way to simulate the natural way of infection compared to injecting way of infection with live borrelia strains.[2] The infectivity of the ticks determined by PCR resulted in 20 % of infective ticks. The number of the attached ticks was 43.1 % after removing the fixation chamber. The fed ticks were later destroyed by the dogs themselves. Application and attachment of the ticks is shown in the pictures (Fig 1., 2.). This application method was shown to be suitable for such purpose and gave good results of attached ticks.[2]

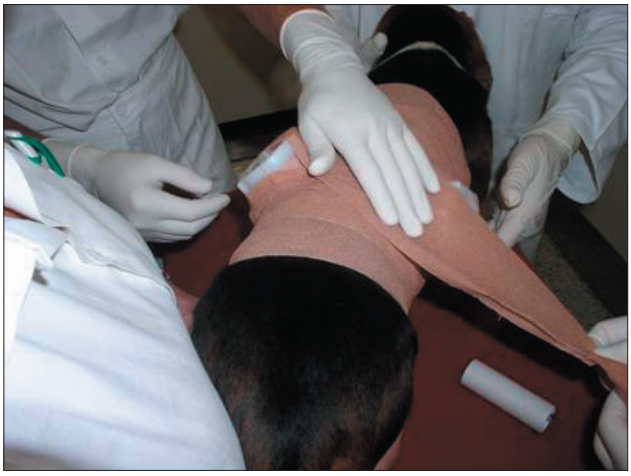

**Fig. 1.** Fixation of the chamber

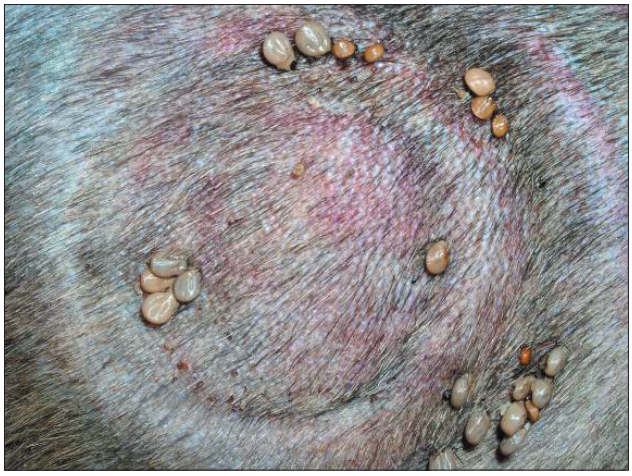

**Fig. 2.** Attached ticks

**Table 1.** Re-isolation of spirochaetes from the skin and post mortem tissue samples. Spirochaetes were detected by growth in cultures from control dog tissues and from two samples of dog No. 12, R – right side, L – left side, K – control dog, – negative and + positive cultivation

| Dog no. | | IN VITRO | | | | POST MORTEM | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Skin 125 days | | Skin 141 days | | Skin 156 days | | | | | |
| 7 | Vaccinated | L | – | L | – | L | – | L | – | L | – |
| | | L | – | L | – | L | – | | | | |
| | | R | – | R | – | R | – | R | – | R | – |
| | | R | – | R | – | R | – | | | | |
| 8 | | L | – | L | – | L | – | L | – | L | – |
| | | L | – | L | – | L | – | | | | |
| | | R | – | R | – | R | – | R | – | R | – |
| | | R | – | R | – | R | – | | | | |
| 9 | | L | – | L | – | L | – | L | – | L | – |
| | | L | – | L | – | L | – | | | | |
| | | R | – | R | – | R | – | R | – | R | – |
| | | R | – | R | – | R | – | | | | |
| 10 | | L | – | L | – | L | – | L | – | L | – |
| | | L | – | L | – | L | – | | | | |
| | | R | – | R | – | R | – | R | – | R | – |
| | | R | – | R | – | R | – | | | | |
| 11 | | L | – | L | – | L | – | L | – | L | – |
| | | L | – | L | – | L | – | | | | |
| | | R | – | R | – | R | – | R | – | R | – |
| | | R | – | R | – | R | – | | | | |
| 12 | | L | – | L | – | L | + | L | – | L | – |
| | | L | – | L | – | L | – | | | | |
| | | R | – | R | – | R | – | R | – | R | + |
| | | R | – | R | – | R | – | | | | |
| 13K | Non-vaccinated | L | – | L | + | L | + | L | + | L | + |
| | | L | – | L | – | L | + | | | | |
| | | R | – | R | – | R | + | R | + | R | + |
| | | R | – | R | + | R | + | | | | |
| 14K | | L | – | L | – | L | + | L | + | L | + |
| | | L | – | L | – | L | + | | | | |
| | | R | – | R | + | R | + | R | + | R | + |
| | | R | – | R | + | R | + | | | | |

During the two months of monitoring after the challenge, no significant clinical symptoms (apathy, dysorexia, limping, crisp hair, touch painful) were observed at the vaccinated or control dogs. This fact can be caused by the relatively short period of the monitoring after the challenge with respect to the clinical symptom manifestation.[2]

The detection of borreliae by using the method of cultivation in BSK-H medium was shown in both control dogs before post-mortem examination from skin biopsies and in all samples tested post-mortem. From the vaccinated dogs, borreliae were cultivated only from one dog in the post-mortem examination, i.e. from the skin biopsy (left side) and from the muscle biopsy (right side) only. The results are summarized in Tab. 1.

On the other hand, PCR detection did not prove the penetration into the tissues of control or vaccinated dogs. It is probably caused by limitation of the classical PCR method and limited amount of borreliae able to penetrate into different tissues.[9,22]

Protection of the vaccinated dogs against infectious agents during the challenge experiment was examined also by the serological tests.[8] Examined sera of the vaccinated dogs contained high titer of anti borrelia IgG specific antibodies, which reached the maximum titer between 21st and 49th day after vaccination and then slowly decreased (Fig. 3).

As the most important serological examination is considered the determination of high titer of OspA antibodies, which are known to be the most important for the protec-

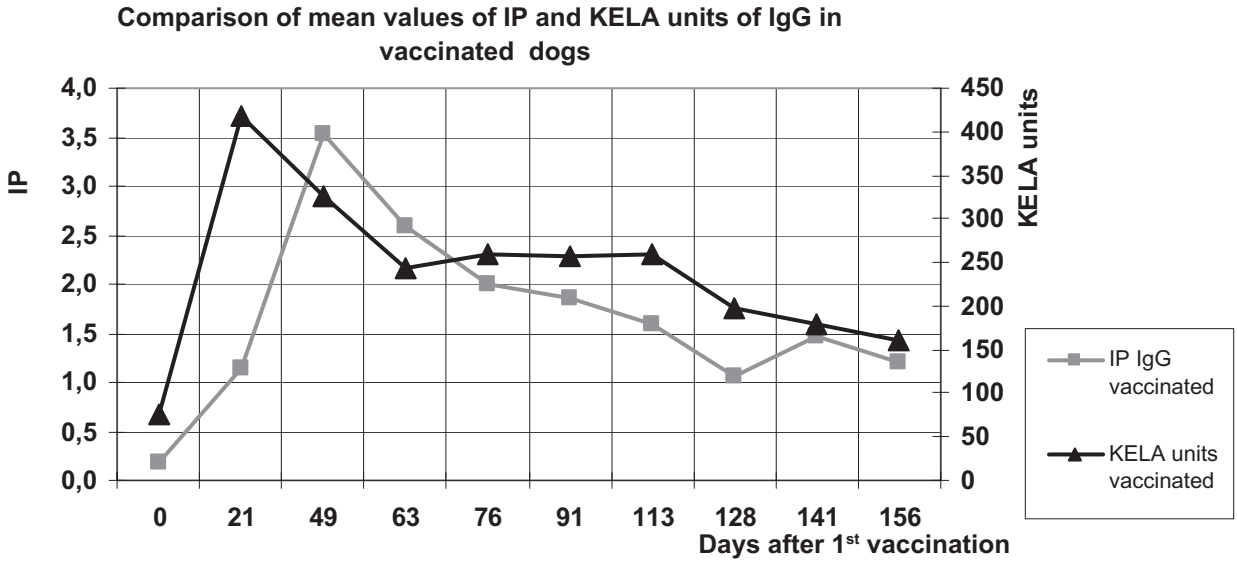


**Fig. 3.** The shapes of the curves, of mean values index positivity of IgG measured with TEST-LINE commercial kit (squares) and ELISA KELA (triangles) unit measured by Straubinger, are almost similar. The high titer levels of specific borrelia IgG antibodies persisted for three months.

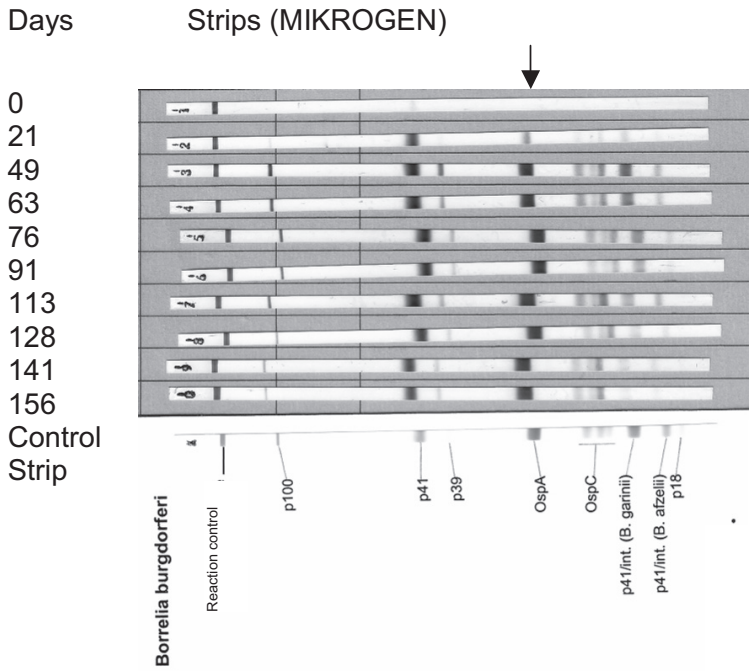


**Fig. 4.** The formation and persistence of specific OspA IgG antibodies during the challenge experiment (sera sample of vaccinated dog No. 9). The challenge was performed on 101st day. The arrow indicates location of OspA protein structure on the strips.

tion against borreliae infection.[11,13,23] The examination was also supplemented with determination of OspA protein presence in the production strains (*B. afzelii*, *B. garinii*), which are used for preparation of the vaccine Biocan® B inj. ad us. vet. inj. ad. us. vet., the results conform (data not shown). Sera of the vaccinated dogs contained high titer of OspA IgG antibody shown with MIKROGEN Western blots. The highest titer was seen one month after revaccination. The OspA antibodies of dog No.12 were weaker, which was in accordance with positive cultivation of spirochaetes.

All these results, in total, furnish the proof of protective activity of the dogs vaccinated with Biocan® B inj. ad us. vet. against borreliosis.

The development of the new recombinant vaccine is based on preparation of recombinant proteins of the borrelia surface structures. Formation of specific recombinant proteins IgG antibodies in mice was confirmed by

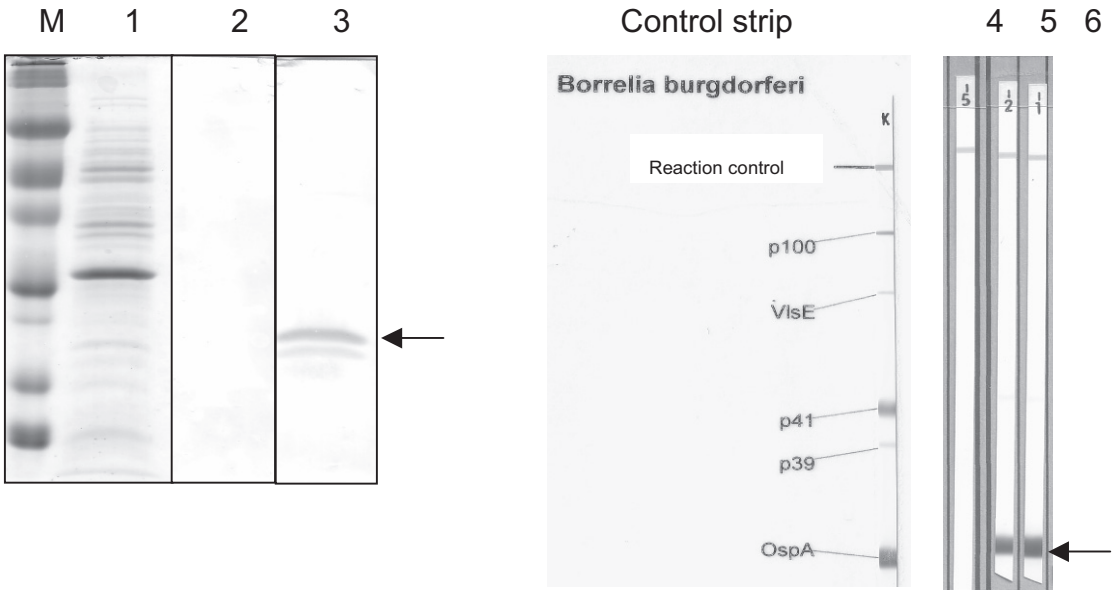


**Fig. 5.** Formation of rOspC/BG, rOspA/BA and rOspA/BB antibodies. SDS electrophoresis of *B. garinii* antigen structures which were transferred to membrane, line M – marker, line 1 – *B. garinii*, line 2 – initial sera, line 3 – sera of mice immunizer with rOspC/BG. Formation of rOspA antibodies using MIKROGEN strips with OspA structures, line 4 – initial sera, line – 5 sera of the immunized mice with rOspA/BA, line 6 – sera of the immunized mice with rOspA/BB. Arrows indicate OspC and OspA proteins positions.

Western blots (Fig 5). These proteins are suitable for following serological and challenge tests and they are candidates for subsequent preparation of the new recombinant vaccine.[15, 20, 23]

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