DEVELOPMENT AND IMMUNOGENIC PROPERTIES
OF EXPERIMENTAL VACCINE
AGAINST LYME DISEASE IN DOGS

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Variability of outer surface proteins described among different genospecies of Borrelia burgdorferi is responsible for the lack of full protection of vaccinated dogs against challenge with heterologous spirochete strains. Due to that, the incorporation of multiple isolates or protein subunits may be necessary to increase the effectiveness of the vaccines. An experimental vaccine developed by the authors contained 4 strains of B. burgdorferi representing genospecies of spirochete prevalent in Poland. IgM antibodies of titre 200/400 were demonstrated in all vaccinated puppies, on day 7 after immunization. Their titre was increasing up to day 14 reaching the value of 800/1600. Between day 35 and 42 after vaccination the level of IgM antibodies decreased to 400. IgG antibodies of titre 200 were demonstrated in all vaccinated animals on day 7. Their level increased between day 7 and 21 after immunization up to 400/800 and then decreased to the value of 200/400, despite of the revaccination of animals. The results of the presented studies confirmed safety and immunogenic properties of an experimental vaccine against B. burgdorferi infection in dogs.

Key words: dogs, Lyme disease, experimental vaccine.

Lyme disease, caused by the spirochete B. burgdorferi is the most common tick-borne zoonosis occurring in humans and dogs. The disease is multisystemic, characterized by early skin lesions and late arthritis, cardiac and neurologic manifestations. In dogs, limb and joint disorders associated with borreliosis can result in gait abnormalities and reluctance to move. Renal, cardiac and neurological disorders occur less frequently in canines. Other clinical signs include fever, lethargy and loss of appetite (1, 12, 20). At least 10 genospecies of Lyme disease spirochetes have been recognized on the basis of genetic and molecular determinants. Published data indicate the geographical distribution of different genospecies of the spirochete. B. burgdorferi sensu stricto is prevalent in the United States and Europe, but it is not reported in Asia. B. garinii and B. afzelii are demonstrated in Europe and Asia, but they are not observed in the USA. In Poland four genospecies of B. burgdorferi were identified on the basis of results of preliminary studies carried out in the Department of Carnivores and Fur Animal Diseases of the National Veterinary Research Institute: B. burgdorferi sensu stricto, B. afzelii, B. garinii and B. lusitaniae (17).
Commercially available vaccines against Lyme disease for dogs contain inactivated strains of *B. burgdorferi*. In different laboratories studies on the application of molecular biology methods to develop recombinant proteins of the spirochete, mainly outer surface proteins (2, 3, 4, 6, 8, 15, 19) or DNA vaccines are carried out (13, 23).

Variability of outer surface proteins described among different genospecies of *B. burgdorferi* is responsible for the lack of the full protection of vaccinated dogs against challenge with heterologous spirochete strains (6, 24). Due to that, the incorporation of multiple isolates or protein subunits may be necessary to increase the effectiveness of the vaccines. Elaboration of an experimental vaccine containing inactivated spirochetes of four genospecies of *B. burgdorferi* prevalent in Poland was the aim of the presented studies.

**Material and Methods**

*B. burgdorferi* strains used for preparation of the vaccine. Previous experiments performed in the Department of Carnivores and Fur Animal Diseases of the National Veterinary Research Institute revealed that the following genospecies of *B. burgdorferi* are prevalent in Poland: *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii* and *B. lusitaniae* (17). The following strains of spirochete representing four genospecies were used for vaccine preparation: reference strains - *B. burgdorferi* sensu stricto - B31, *B. garinii* – 20047 and *B. afzelii* - VS461 as well as Polish tick isolate - 236/2 representing *B. lusitaniae*.

Cultivation of *B. burgdorferi* sensu lato in BSK-H medium. Four strains of *B. burgdorferi* selected for the vaccine preparation were cultivated in BSK-H medium (Sigma) supplemented with 6% of rabbit serum and antibiotics (Sigma), at 31°C for 14 - 21 d. Bacterial growth was monitored by dark field microscopy. The consecutive passage of spirochetes was done when their density was estimated as ++++, corresponding to spirochete concentration of ca 2x10^8/ml. After the third passage, the density was evaluated in spectrophotometer (Carl Zeiss, Jena), with the wave length of 600 nm. Required OD_600 value was 0.15, which corresponded to 10^9 spirochetes/ml.

Stability of the strains used. Stability of the spirochetes used for vaccine preparation after 20 passages in BSK-H medium was confirmed by amplification of SS-23S intergenic spacer according to the method described earlier (17) and restriction enzyme analysis of amplicons with the use of endonucleases MseI and DraI. Restriction patterns were compared with the use of the computer programme BIO1D (Vilber-Lourmat, France).

Preparation of the experimental vaccine. Cultures of *B. burgdorferi* with the density of 10^9 spirochetes/ml were centrifuged at 4000 rpm (MPW-360 centrifuge) for 10 min. Supernatants were discarded and pellets were washed twice with sterile PBS. After the last washing step the spirochete pellets were collected and resuspended in sterile PBS up to the final volume of 313.76 ml. For inactivation of the spirochetes, 36% formaldehyde in the final concentration equal to 0.2% was used. Inactivation step was carried out at 37°C for 24 h. After inactivation, 55.5 ml (15% v/v) of ALHYDROGEL - Aluminium Hydroxide Gel Adjuvant (Superfos, Denmark) was added. The experimental vaccine was stored at 4°C.
Bacterial purity tests. Experimental vaccine was submitted for bacterial purity tests according to the standard procedures. Two hundred µl of the vaccine were inoculated into each medium. All samples were incubated at 37°C and in case of Sabouraud’s medium at room temperature for 14 – 21 d.

Evaluation of the efficacy of the inactivation process. Two hundred µl of the vaccine were inoculated into 2 ml of BSK-H medium (Sigma) supplemented with 6% of rabbit serum (Sigma) and antibiotics (Sigma). The sample was incubated at 31°C for 14 d and then examined in the dark field microscope under the 100x magnification.

Evaluation of the safety of the vaccine. Safety of the vaccine was tested on laboratory animals by injecting subcutaneously 8 mice (0.25 ml of the vaccine), 6 guinea pigs (0.5 ml) and 4 dogs (2 dogs – 2 ml of the vaccine and 2 dogs – 5 ml of the vaccine). All animals were observed for the presence of local and general reactions for 14 d after inoculation of the vaccine.

Vaccination scheme. Ten mixed-breed dogs of both sexes, aged 10-12 weeks, with the weight of 4-5 kg were used in the studies. The dogs were housed in individual cages and fed a commercial diet. Eight puppies received two doses of the vaccine given on days 0 and 21: four dogs - 1 ml/dose administered subcutaneously and four puppies – 1 ml/dose administered intramuscularly. Two animals served as non-vaccinated controls.

Safety monitoring in vaccinated puppies. The rectal temperature of all vaccinated and control animals was recorded five days before the vaccine administration, on vaccination day and during five days after immunization. Hyperthermia was defined as a rectal temperature > 39.5°C. Local and general reactions in vaccinated animals were recorded during 14 d following vaccination.

Evaluation of immunogenic properties of the experimental vaccine. Studies on immunogenic properties of the vaccine were carried out on dogs free from antibodies specific to B. burgdorferi. Blood samples for serological examinations were collected from vaccinated animals on day 0 and 7, 14, 21, 28, 35 and 42 d after immunization. The level of antibodies specific to B. burgdorferi was evaluated with the use of Canine B. burgdorferi Antibody Test Kit (IDEXX) and Dog EIA Borrelia IgG/IgM (TestLine).

Results

Restriction enzyme analysis of intergenic spacer amplicons (ISA) of the strains used for the vaccine production, with the use of endonucleases MseI and DraI confirmed the stability of the spirochetes after their 20 subsequent passages in BSK-H medium (Figs 1 and 2). The sizes of fragments obtained as a result of enzymatic cleavage were in accordance to those described earlier by Postic et al. (18). The laboratory control of the experimental vaccine did not reveal any microbiological contaminations. The efficiency of the inactivation process was confirmed by dark field microscopy. The examination of the samples of BSK-H medium inoculated with 200 µl of the vaccine, carried out on days 14, 21 and 42 of the incubation at 31°C did not reveal the presence of the viable spirochetes. All of the tissue cultures were considered sterile, although parallel media controls did support the growth of various laboratory strains of B. burgdorferi.
Fig. 1. Restriction fragments obtained after enzymatic cleavage with the use of \textit{MseI}: 1- pUC19 \textit{HaeIII/ExpI} (InGen); 2- \textit{B. burgdorferi ss B31}; 3- \textit{B. garinii} 20047; 4- \textit{B. afzelii} VS 461; 5- \textit{B. lusitaniae} 236/2.

Fig. 2. Restriction fragments obtained after enzymatic cleavage with the use of \textit{DraI}: 1- pUC19 \textit{HaeIII/ExpI} (InGen); 2- \textit{B. burgdorferi ss B31}; 3- \textit{B. garinii} 20047; 4- \textit{B. afzelii} VS 461; 5- \textit{B. lusitaniae} 236/2.
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* - revaccination;  
++ - high level of antibodies in IDEXX test

Table 1
Immunogenic properties of the experimental vaccine for dogs
In vaccinated laboratory animals transient local reactions were observed in 2 mice and 1 guinea pig. Slight, transient nodules were reported in puppies immunized with 5 ml doses of the vaccine in safety studies. No general reactions were observed in puppies vaccinated with 1 ml of the vaccine during 14 d of observation period. Only one pup demonstrated the transient nodule on the left side at the site of injection of the vaccine given subcutaneously and slight hyperthermia two h after the first vaccine injection. No hyperthermia was observed in other pups.

Results of serological examination of vaccinated and control dogs are presented in Table 1. Antibodies specific to *B. burgdorferi* were demonstrated by Canine *B. burgdorferi* Antibody Test Kit 14 d after vaccination in all immunized dogs. The level of antibodies designated by the Producer as high ("++") was maintained for 42 d after vaccination. Quantitative analysis of antibodies performed with the use of Dog EIA *Borrelia* IgG/IgM revealed the presence of IgM antibodies of titre 200/400 in all vaccinated puppies on day 7 after immunization. Their titre was increasing up to day 14 reaching the value of 800/1600. Between day 35 and 42 after vaccination the level of IgM antibodies decreased to 400. IgG antibodies of titre equal to 200 were demonstrated in all vaccinated animals on day 7. Their level increased between day 7 and 21 after immunization up to 400/800 and then decreased to the value of 200/400, despite of the revaccination of animals. In control, non-vaccinated puppies, antibodies specific to *B. burgdorferi* were not detected. The correlation between the tests used was established. The titre of 100 and 200 in TestLine corresponded to "+" in IDEXX while titre higher than 200 in TestLine was evaluated in IDEXX as high positive ("++"). Results of the presented studies confirmed safety and immunogenic properties of the experimental vaccine against *B. burgdorferi* infection in dogs.

**Discussion**

During the last decade investigations of immunoprotective antigens of Lyme disease spirochetes and the development of safe and efficacious vaccines have been the focus of the research (2, 3, 4, 6, 8, 9, 19, 21). Killed whole cells of *B. burgdorferi* were first used as a vaccine in hamsters (10). Also commercial vaccines for dogs, including those available in Poland, contain inactivated one of *B. burgdorferi* strains. Recently, the most promising vaccine candidates are the major outer surface proteins that are expressed in abundant amounts on the outer membrane of the spirochete (2, 3, 4, 6, 13). It is known that the proteins provide full or partial protection in experimental animals. However non-surface fusion protein with a molecular mass of 110 kDa containing a truncated HSP70 as well as P39 (BmpA) may also be protective (8, 22). Studies of Hughes et al. (8) with the use of avirulent *B. burgdorferi* 297 mutant that lacked the plasmid encoded outer surface proteins OspA, OspB and two lipoproteins of 20 and 7.5 kDa (that were observed in the wild type) suggested that OspC and/or P39 are important for the development of protective immune response. However, immunogenic properties of P39 were not confirmed by Gilmore et al. (7). The examination of sera from dogs vaccinated with *B. burgdorferi* bacterin done by Magnarelli et al. verified the presence of antibodies to OspA, OspB, p22, p41-G and p37 in vaccinated animals, while there was frequent reactivity to the P39 antigen after natural infection (16).

Studies of Ma et al. (14) demonstrated that a QS21-formulated recombinant FLOspA and FLOspB-based vaccine could elicit a maximal humoral response in dogs, but the antibody response was against a homologous *B. burgdorferi* sensu stricto B31
isolate and a heterologous isolate CA-2-87 (California isolate), but not against 2 European isolates of French and Swedish origin. It is possible that weakly immunogenic antiborrelial epitopes may be conserved on OspA and OspB. In particular, OspA-vaccinated animals were not completely protected against needle-administered challenge with heterologous strains. Also results of the experimental work carried out on dogs by Jobe et al. (9) suggested that vaccination with canine Lyme disease vaccine could provide protection against infection from some B. burgdorferi isolates, primarily those related to isolate used for vaccine preparation. They also demonstrated that vaccination did not induce the protection against the European genospecies B. garini and B. afzelii nor against an isolate representing another U.S. seroprotective group. Those findings were also confirmed by other researchers who suggested that variability of lipoprotein OspA described among different genospecies of B. burgdorferi and within isolates of B. burgdorferi sensu stricto is responsible for the lack of the full protection of vaccinated dogs against challenge with heterologous strains of the spirochete (6, 24). In Europe, the variability of OspA among B. burgdorferi sensu lato isolates is often used as an argument against OspA vaccine. Considering this fact, the incorporation of multiple isolates or protein subunits may be necessary to increase the effectiveness of the vaccines. In our experimental inactivated vaccine for dogs we incorporated 4 isolates of B. burgdorferi representing genospecies of spirochete prevalent in Poland. We used aluminium hydroxide, an adjuvant commonly used for preparation of human and animal vaccines. According to data published by Erdile et al. (5) and Ma et al. (14), this adjuvant is unable to significantly increase the induction of functional antibody response to OspA. These findings were not confirmed in our studies because the highest level of antibodies induced in dogs by experimental vaccine was equal to 1600. This can be also due to highly immunogenic nature of lipoprotein A of the spirochete.

The safety and efficacy of a commercially available B. burgdorferi bacterin were examined by Levy et al. (11) under field conditions on 1969 dogs that received a total of 4 033 doses of the vaccine during a 20-month period. B. burgdorferi bacterin was found to be safe and efficacious under field use. Postvaccination reactions were minor, developed infrequently and resolved without complications. Similar findings were reported in our studies. No general reactions were observed in puppies vaccinated with 1 ml of the vaccine during 14 d observation period. Only one pup demonstrated the transient nodule on the left side at the site of injection of the vaccine given subcutaneously and slight hyperthermia two hours after the first vaccine injection. No hyperthermia was observed in other pups. Postvaccination reactions may be a result of trauma of injection, non-specific reaction to adjuvant or vehicle in the vaccine, non-specific reaction to the primary antigen in the vaccine or specific allergic or immune-mediated reaction to any of the vaccine components.

Results of the presented studies demonstrated the safety and immunogenic properties of the experimental vaccine for dogs, but further experiments are needed to elaborate recombinant vaccine which could be applied both for humans and animals.

References


