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A STUDY ON THE INACTIVATED BIVALENT VACCINE PREPARED FROM SEROTYPES 1/2a AND 4b *Listeria monocytogenes* FOR THE CONTROL OF LISTERIOSIS IN SHEEP

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In this study, the protective effects of two bivalent inactivated vaccines were evaluated. Vaccines were prepared from Listeria monocytogenes, serotypes 1/2a and 4b, as the most frequent in our and surrounding epidemiological areas. Vaccine A consists of whole L. monocytogenes bacteria cells, inactivated with 0.4% formaldehyde and aluminium hydroxide as a carrier. Vaccine B contains 0.1% saponin in addition to ingredients of vaccine A. Evaluations of these vaccines were performed in 60 sheep, divided into four groups (n=10) with a corresponding negative control group (n=5). After 14 days, boosterisation of all animals was performed. In order to evaluate the immune response, blood samples were obtained every 14 days during the next 6 months. Antibody titres were determined by microaglutitation (MAT) and complement fixation tests (CFT).

Comparative analyses of antibody titres, induced by vaccines A and B, show that the latter (with saponine) significantly increased the level of antibody titres ($p<0.01$). The levels of immune response were also significantly impacted by the total number of bacteria and vaccine dosage ($p<0.01$).

The bivalent vaccine containing 0.1% saponin (vaccine B) in 5.0 mLx 10^6 cfu/mL (colony-forming units per milliliter) dosage shows a protective effect after challenge with L. monocytogenes. The protective levels of this antibody were 1/80 and 1/16, determined by MAT and CFT, respectively.

Antibody titres were significantly higher after boosterisation ($p<0.01$) and protective levels could be detected in the sera of vaccinated animals during the next 6 months. Therefore, it is strongly recommended to perform boosterisation two weeks after the initial vaccination.

Key words: Listeria monocytogenes, vaccine, saponin, sheep

INTRODUCTION

Listeria monocytogenes, a gram-positive, facultative intracytosolic bacteria, is the causative agent of listeriosis, a severe disease associated with a high mortality rate. Nearly all domestic animals are susceptible to *L. monocytogenes* infection, but animal listeriosis most commonly occurs in ruminants. Listeriosis is also an important food-borne zoonosis (Farber and Peterkin, 1991; Regan *et al*., 2005). In ruminants, among which sheep are the most commonly affected, listeriosis is usually represented by three distinct clinical syndromes; septicemia, encephalitis (meningoencephalitis), and abortion. Ovine listeriosis has also been associated with mastitis, ophthalmic lesions such as keratoconjunctivitis and gastroenteritis (Wagner *et al*., 2000; Clark *et al*., 2004; Otter *et al*., 2004; Gasanov *et al*., 2005).

Listeria is widely distributed in the natural environment and has been isolated from the soil surface, rotten vegetables and pasture herbage. Domesticated ruminants play a key role in the maintenance of *Listeria* spp. in the rural environment through a continuous faecal–oral enrichment cycle (Picoux, 2008). The main source of infection in ruminants is a poorly fermented silage with a pH above 5.0–5.5 and with pockets of aerobic deterioration (microaerophilic microorganisms). In general, the disease is more frequent in winter and early spring. The risk during the winter season is associated with the fact that *Listeria* can grow at temperatures where growth of other pathogens is inhibited due to excessive cold (psychrophilic microorganisms) (Radostits *et al*., 2007).

L. monocytogenes can be distinguished in 16 serovars on the basis of somatic and flagellar antigens and there is genetic diversity between serovars (Beumer and Hazeleger, 2002). Two virulent serovars (I/2a and 4b) are most commonly isolated in farm animal diseases in Serbia (unpublished data). These strains can multiply in macrophages and monocytes and produce a haemolysin, listeriolysin O (LLO), which is the virulence determinant of the organism (Bennett and Weaver, 2001; Amagliani *et al*., 2006).

The recovery rate is higher if treatment is administered early in the course of the disease. Antibiotics (ampicilline and gentamicin) must be administered for a prolonged period, because recovery can take as long as one month (Low and Donachie, 1997). Treatment of severely ill or recumbent animals with listeriosis is rarely successful (George, 2002). Immunoprophylaxis (i.e. vaccination) has proved itself over the past 120 years to be by far the most efficient and cost-effective method of controlling infectious diseases. Nevertheless, vaccination is not always an innocuos procedure, and its use must always be accompanied by a careful assessment of the risks and benefits of the procedure (Tizard, 2006).

Taking into account all of the above mentioned, the objective of this study was to evaluate the protective effects of two inactivated, bivalent vaccines prepared from two of the most frequent serotypes of *L. monocytogenes* in our and surrounding epidemiological areas.

MATERIAL AND METHODS

Isolation and serotypization of isolated L. monocytogenes strains

L. monocytogenes was isolated from samples of aborted fetuses or brains of dead sheep on tryptone agar (Torlak, Belgrade-Serbia) according to the standard procedure. Serotypization of the isolated strains was determined by the method of fast agglutination on a microscope slide coated with anti-O and anti-H immunoserum of *L. monocytogenes* (Bioveta, Czech Republic).

Preparation of vaccines

Vaccines were prepared from whole *L. monocytogenes* bacterial cells, serotypes 1/2a and 4b, inactivated with 0.4% formalin with the addition of aluminium hydroxide as a carrier (vaccine A). Vaccine B, besides the above mentioned constituents it contained 0.1% saponin, also.

Examining the sterility and toxicity of vaccines

Examination of the sterility of the prepared vaccines was performed by sowing on nutritive agar, 5% blood agar and tryptone agar. During the five day incubation period (37^{o}C), the substrates were inspected on a daily basis, no growth of aerobic and anaerobic microorganisms was detected.

The toxicitiy of vaccines was also examined on laboratory mice. Briefly, after subcutaneous application of the examined vaccine, the dose being 0.3 mL, the mice were monitored daily for any possible changes in the general condition during a period of four weeks. After this period, the mice were sacrificed and a suspension of parenhimatic organs was sown on tryptone and blood agar. During this period of monitoring, no deaths of mice were recorded.

Serological reactions

Titre determination of the antibodies after immunization was performed by the method of slow agglutination (MAT), according to the instructions of the manufacturer (Bioveta, Czech Republic) and by the complement fixation test (CFT). For serological reactions, personally prepared and standardized antigenes of *L. monocytogenes*, serotypes 1/2a and 4b were used.

Examination of the prepared vaccines on sheep

The examination of vaccines (A and B) was performed on 60 sheep of the Würtenberg breed aged between three and four months, with a average body mass of 30-35 kg. The sheep were divided into four groups (n=10), each group having its own negative control group (n=5). Before applying the analysed vaccines, subcutaneously into the knee fold, blood samples were obtained by puncture of *v. jugularis* with the aim of detecting the possible presence of antibodies of the studied *L. monocytogenes* serotypes. The first group of animals was vaccinated with 2.5 mL $\times$ 10^6 cfu/mL, the second group with 5.0 mL $\times$ 10^6 cfu/mL of vaccine A. The third group was vaccinated with 2.5 mL $\times$ 10^6 cfu/mL, while the fourth group received 5.0 mL $\times$ 10^6 cfu/mL of vaccine B, respectively.

Sheep from the negative control groups received subcutaneous injections of physiological solution (Hemofarm, Vršac).

Two weeks after immunization, the sheep were revaccinated according to the same protocol. Determination of the antibody titre in the blood serum after immunization was performed at fortnightly intervals, commencing from the second week and ending on the 24th week.

All experimental procedures were performed according to our insitutional guidelines for animal research and principles of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other (Official Daily N. L 358/1–358/6, 18, December 1986).

Statistical analysis

Statistical significance of differences of all examined parameters were determined by means of ANOVA, followed by Tukey test. Data were expressed as means ± standard error. The level of significance was set at $p<0.05$. Statistical analysis was performed using the Graph Pad Prism 5.0 Software, CA, USA.

RESULTS

By examination of the blood serum on day zero, (before commencing with the vaccination) and by employing the method of slow agglutination, it was determined that the sheep included in the experiment were seronegative for 1/2a and 4b *L*. *monocytogenes* serotypes.

Results of sheep antibody titres obtained by the method of microagglutination

Titres of agglutinized antibodies in sheep vaccinated with vaccine A ranged within the interval from 1/20 to 1/80, while in sheep which received vaccine B, the titres ranged from 1/40 to 1/320 (results not shown).

It has been determined by statistical analysis that the average antibody titre was highest in group IV which received vaccine B, the dose being 5.0 mL (17.69± 1.11), while the lowest titre was in group I which received vaccine A, the dose being 2.5 mL (12.96 ± 1.25) (Figure 1). Comparing the average values of antibody titres, a high statistical difference was determined between all the studied groups ($p<0.01$). Comparing antibody titre values between the groups that received different doses of vaccine B, no statistical differences were detected ($p>0.05$).

Analyzing the trend of agglutinized antibody titre movement, after applying vaccine A in a dose range from 2.5 to 5.0 mL during 24 weeks, it has been detected that the titre gradually increases, reaching maximum values on the sixth and fourth week respectively, followed by a fall (Figure 2). A very similar tendency is for antibody titre after the application of vaccine B with a dose of 2.5 mL. The titre gradually rises and reaches its peak on the eigth week after vaccination, followed by a downfall (Figure 2).

Analyzing the tendency of agglutinizing antibody titre movement after application of vaccine B, with doses of 2.5 and 5.0 mL, during 24 weeks, it was determined that the titre gradually increases, reaching its maximum value on the

sixth week followed by a gradual fall (Figure 2). A very similar trend is shown by the antibody titre after application of vaccine B in a dose of 2.5 mL. The titre gradually rises, reaching its highest level on the eigth week after vaccination, followed by a gradual fall (Figure 3).

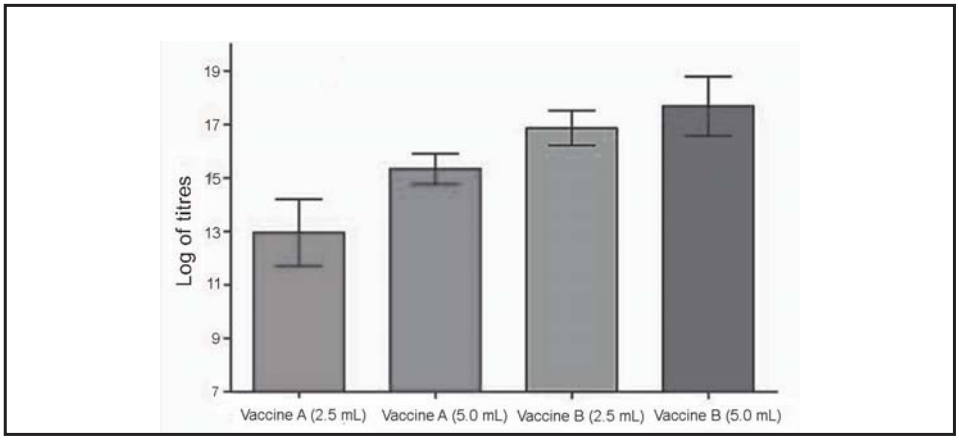

Figure 1. Average titres of antibody following the application of vaccines A and B in different dosages, determined by MAT, Group I versus group II, $p<0.01$; Group II versus group III, $p<0.01$; Gruop III versus group IV, $p>0.05$

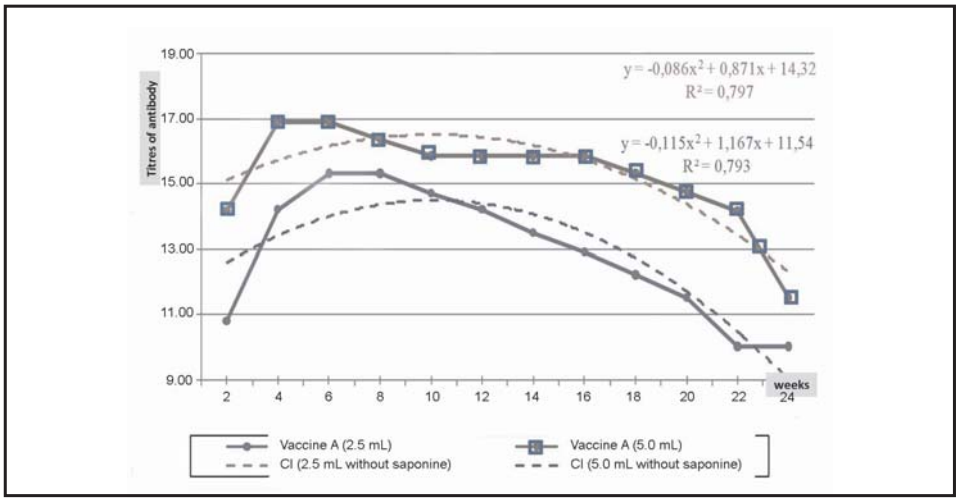

Figure 2. The trend of agglutinized antibody titres movement during 24 weeks period following the application of vaccine A in 2.5 and 5.0 x 10^6 cfu/mL, determined by MAT, CI – Confidential Interval

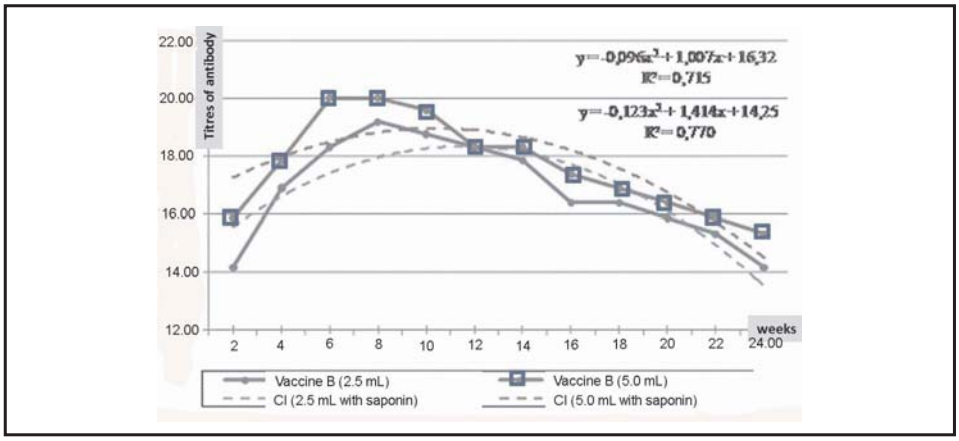

Figure 3. The trend of agglutinized antibody titres movement during 24 weeks period following the application of vaccine B in 2.5 and 5.0 x 10^6 cfu/mL, determined by MAT, CI – Confidential Interval

Results of sheep antibody titres obtained by the complement fixation test (CFT)

The antibody titre obtained by the method of CFT on serotypes 1/2 and 4b in sheep immunized with vaccine A, ranged from 1/4 to 1/16, while this interval was from 1/4 to 1/32 in sheep immunized with vaccine B (results not shown).

Statistical analysis of the results showed that the average titre was highest in group IV of animals receiving a dose of 5.0 mL (1.99 ± 0.11) vaccine B, while the lowest titre appeared in group I which received vaccine A in a dose of 2.5 mL (1.50±0.17) (Figure 4).

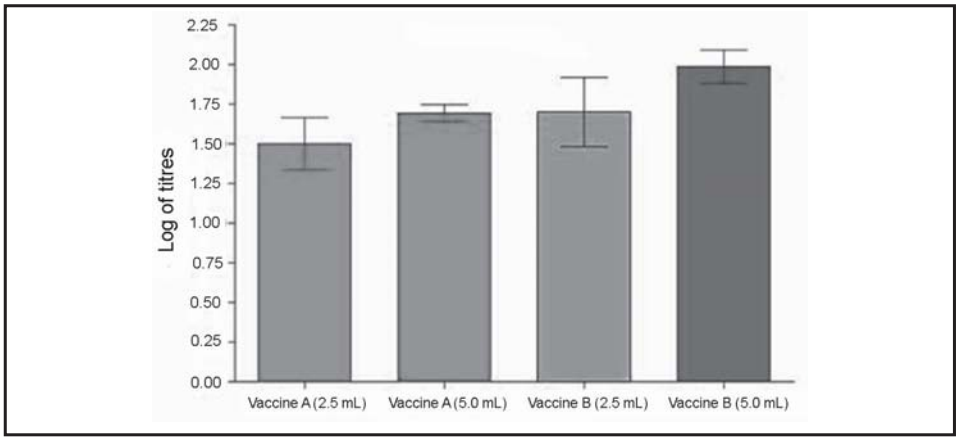

Figure 4. Average antibody titres following application of vaccines A and B in different dosages, determined by CFT, Group I versus group II, $p<0.05$, Group II versus group III, $p>0.05$, Gruop III versus group IV, $p<0.01$

Comparing the antibody titres obtained by the method of CFT, it has been determined that there is a statistically highly significant difference ($p<0.01$) after the application of the studied vaccines in relation to vaccine B applied at a dose of 5.0 mL.

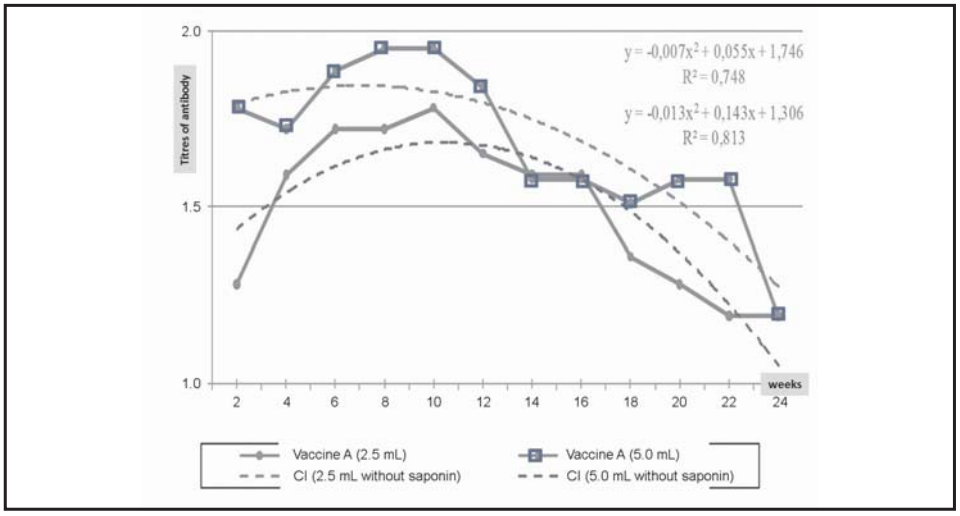

Figure 5. Trend of agglutinized antibody titres movement during 24 weeks period following the application of vaccine A in 2.5 and 5.0 x 10^6 cfu/mL, determined by CFT, CI – Confidential Interval

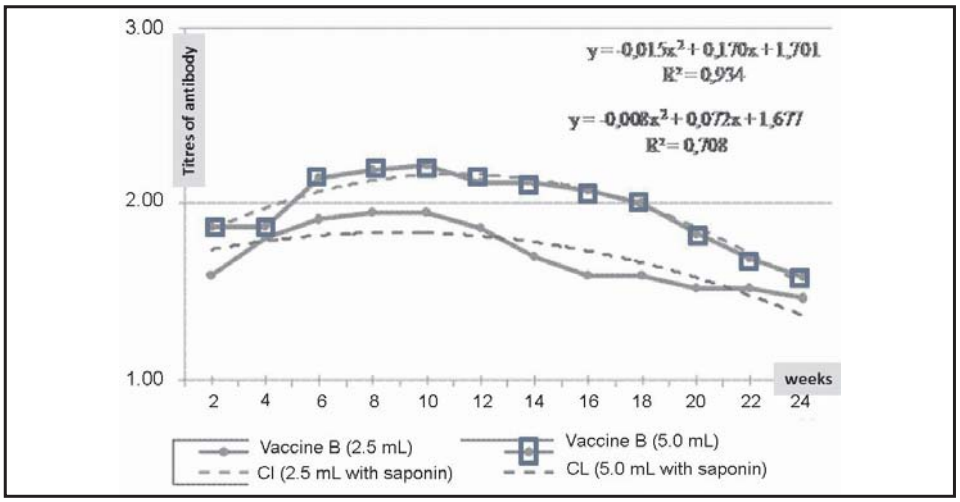

Figure 6. The trend of agglutinized antibody titres movement during 24 weeks period following the application of vaccine B in 2.5 and 5.0 x 106 cfu/mL, determined by CFT, CI – Confidential Interval

By analyzing the trend of movement of the complement fixed antibody titre, after application of vaccine A with doses from 2.5 and 5.0 mL during 24 weeks, it has been determined that the titre gradually increases, reaching its maximum values on the eigth and tenth week respectively, followed by a gradual fall (Figure 5). After applying vaccine B at doses of 2.5 and 5.0 mL, the complement fixed antibody titre gradually rises, reaching the peak on the tenth week (Figure 6).

Results of artificial infection

Artificial infection was performed in five sheep from group IV with antibody titre ranging within average values (1/80-MAT and 1/16 –CFT). Sheep were infected with 5.0 mL x 10^6 cfu/mL of fresh bujon, 24 h culture containig 1/2a and 4b strains, injected subcutaneously in the knee fold. The animals were monitored daily the next 30 days for possible changes in their general health conditions. In the studied sheep, neither body temperature increase nor changes in the general health have been recorded. Bacteriological analyses of fecal samples, chemoculture of blood, nasal and buccal smears were negative for the presence of the studied *L. monocytogenes* strains.

DISCUSSION

Vaccination is a very useful tool in the prevention of infectious diseases, if there is an elicitation of effective, protective antibodies. Two major criteria must first be satisfied in determining whether vaccination should be used to control a specific disease. First, it must be established that the immune system can protect against the disease in question. Second, before using a vaccine, we must be sure that the risks of vaccination do not exceed those associated with the chance of contracting the disease itself (Tizard, 2006; Radostits *et al*., 2007).

Since therapy of listeriosis is currently under discussion and does not always offer good results, listeriosis being a serious zoonosis (Braun *et al*., 2002), the aim of this study was to evaluate the efficiency of our experimental inactivated vaccine in contributing to the control and suppression of listeriosis. On the basis of isolation and serotypization, it has been determined that serotypes 1/2a and 4b *L. monocytogenes* dominate in our epizootological region, as well as in the surrounding countries (unpublished data). This is the reason why in our study we selected the above mentioned *L. monocytogenes* strains.

The key factors for vaccine quality and adequate immune response are: the number of microorganisms in the vaccine, degree of attenuation or means of inactivation, combination of bacterial antigenes in the vaccine, as well as the type of added adjuvant, time of vaccination and revaccination (Gudding, 1989).

It was established that the total number of microorganisms in the vaccine, as well as the vaccine dose, have an effect on the immune response (Linde et al., 1995). For this reason, the animals in our experiment were vaccinated with various doses of vaccine, 2.5 and 5.0 ml $\times$ 10^6 cfu/ml. On the basis of the fact that we used pathogen *L. monocytogenes* serotypes, their inactivation was achieved with formalin with the addition of aluminium hydroxide as the carrier (Szemeredi and Padanyi, 1989).

We determined that the antibody titre is significantly higher if the studied vaccine is applied as a dose of 5.0 mL × 10^6 cfu/mL, in relation to the dose of 2.5 mL × 10^6 cfu/mL (Figures 1 and 3). Comparing antibody titres in animals immunized with vaccines A and B, it has been determined that the addition of saponin in vaccine B, at a dose of 0.1%, significantly supports the immune response (Lhopital *et al*., 1993; Linde *et al*., 1995). These results are expected due to the fact that saponin is a nonspecific activator of the immune system (Tizard, 2006).

Antibody titres obtained by vaccines with saponin differ significantly when compared to titres obtained by applying vaccines without saponin. Titre levels of agglutinized antibodies after applying the vaccine containing saponin ranged from 1/40 to 1/320, while with vaccines lacking saponin, the values ranged from 1/20 to 1/80. Titre levels of complement fixed antibodies in the case of the vaccine with saponin ranged from 1/4 to 1/32, while in the case of the vaccine lacking saponin, the range was 1/2 to 1/8.

The aim of revaccination is boosterization, ie. to obtain higher antibody titres (Tizard, 2006). In the present study, 14 days after vaccination, a revaccination has been performed. It is evident that the agglutinated and complement fixed antibodies persisted in the serum of animals six months after vaccination, specifically with a higher titre after the application of vaccine B with a dose of 5.0 mL 10^6 cfu/mL (Figures 4, 5 and 6). The vaccinal titre obtained in this way was in the range of 1/80 to 1/320, determined by MAT, ie. 1/8 to 1/32 as determined by CFT. Our results are in agreement with the results of other authors (Ivanov, 1985; Gudding, 1989; Linde, 1995), who suggested revaccination for a more efficient protection from *L. monocytogenes*.

With the intention of checking the quality and efficiency of the prepared vaccine, an artificial infection was initiated (Mencikova *et al*., 1989). Taking into account that by applying vaccine B with a dose of 5.0 mL 10^6 cfu/mL, the highest antibody titre was obtained. The examination was performed on five sheep with average titre levels for this group of animals. By monitoring the health conditions of the studied sheep during the period of 30 days, we determined that the bivalent vaccine prepared from inactivated listeria serotypes with saponin and dose of 5.0 mL × 10^6 cfu/mL, protected the studied animals after artificial infection, the protective titre having a value of 1/80 for MAT, ie. 1/16 applying CFT. These results are in agreement with those previously reported by Nowacki *et al*. (1987) and Tzora *et al*. (1998).

Since the studied vaccine has not shown toxicity and was efficient in the protection of sheep after artificial infection, such a vaccine after additional research and evaluation on a larger number of animals could find its application in the control of listeriosis in flocks where listeriosis appears more frequently resulting in greater economic losses (Ivanov *et al*., 1977; Mencikova *et al*., 1989; Regan *et al*., 2005).

In conclusion, we demonstrated that antibody titres were significantly higher after boosterisation and that protective levels could be detected in the sera of vaccinated animals during the following 6 months. Therefore, it is strongly recomended to perform boosterisation two weeks after initial vaccination.

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ISPITIVANJE INAKTIVISANE DVOVALENTNE VAKCINE PRIPREMLJENE OD SEROTIPOVA 1/2a i 4b *Listeria monocytogenes* U KONTROLI LISTERIOZE KOD OVACA

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i AŠANIN N

SADRŽAJ

U ovom radu ispitivano je protektivno dejstvo eksperimentalno inaktivisane dvovalentne vakcine protiv listerioze ovaca.Vakcina je pripremana od sojeva *L. monocytogenes* 1/2a i 4b koji su najzastupljeniji na našem epizootiološkom području i u zemljama u okruženju. Vakcina A sadrži cele bakterijske ćelije koje su inaktivisane 0,4% formalinom uz dodatak aluminijum hidroksida kao nosača. Vakcina B pored gore navedenih sastojaka sadrži i 0,1% saponina. Ispitivanje ovako pripremljene eksperimentalne inaktivisane bivalentne vakcine protiv listerioze ovaca izvedeno je na 60 ovaca podeljenih u 4 grupe (n=10), pri čemu je svaka grupa imala kontrolnu grupu (n=5). Nakon 14 dana urađena je revakcinacija svih oglednih životinja.

Krv je uzorkovana svakih 14 dana, tokom narednih 6 meseci i praćeno je kretanje titra antitela, metodom spore aglutinacije (MAT) i reakcijom vezivanja komplementa (RVK).

Uporednim ispitivanjem visine titra antitela kod životinja koje su imunizovane vakcinom bez saponina i vakcinom sa 0,1% saponina ustanovljeno je da saponin značajno podstiče imunski odgovor. Ustanovljeno je da ukupan broj mikrooganizama u vakcini, kao i doza vakcine, utiču na kvalitet imunskog odgovora. Utvrđen je viši titar antitela ako se aplikuje doza vakcine od 5,0 ml $\times$ 10^6 cfu/ml nego kada je aplikovana doza od 2,5 ml $\times$ 10^6 cfu/ml ($p<0,01$).

Dvovalentna vakcina pripremljena od inaktivisanih serotipova listerija sa saponinom u dozi od 5,0 ml $\times$ 10^6ml štitila je jagnjad od veštačke infekcije, a protektivni titar iznosio je 1:80 utvrđen metodom mikroaglutinacije, odnosno 1:16 metodom reakcije vezivanja komlementa. Titri antitela nakon revakcinacije su značajno viši nego posle prve vakcinacije (p,01) i mogli su da se otkriju u serumu životinja 6 meseci nakon vakcinacije, zbog čega se preporučuje obavezna revakcinacija 2 nedelje nakon vakcinacije.