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Development and assay of inactivated pasteurella vaccine for rabbits

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The aim of the study was to select strains for inactivated pasteurella vaccine and to develop effective measures for active immunization of rabbits. Eleven strains (14.6%) of *Pasteurella multocida* from 75 clinical samples were isolated. Five strains (45%) of isolated pasteurella belonged to capsular type A, three strains (27%) to capsular type D, and three strains (27%) were acapsulated. One strain of capsular type A and one strain of capsular type D of *P. multocida* were used for vaccine development. The oil-in-water adjuvant "Emulsigen" (MVP laboratories, Inc., USA) was used.

A laboratory trial of the vaccine against rabbit pasteurellosis, using rabbits as experimental and at the same time as target animals, revealed that the percentage of survivals was 100% to *Pasteurella multocida* infection when animals were vaccinated by not less than 4×10^9 bacterial cells injected subcutaneously. The ratio of both pasteurella capsular types in the vaccine was 1:1 and it appeared suitable for vaccine production. The vaccine was safe and had no side effects on rabbits, except slight swelling at the site of injection. The results obtained after immunization of rabbits in pasteurella-affected rabbit farms were also satisfactory. After immunization, signs of respiratory disorders in rabbits significantly decreased.

Key words: *Pasteurella multocida*, rabbits, vaccine, immunization

INTRODUCTION

Pasteurella multocida is a well-known cause of morbidity and mortality in rabbits. In most cases pasteurella appears in the respiratory tract of rabbits. The predominant syndrome is the upper respiratory disease or "snuffles" [1]. Other local infections are also frequent. *Pasteurella multocida* can cause abscesses, mastitis, otitis and wound infections, it may colonize the paranasal sinuses, middle ears, lacrimal ducts, thoracic organs, and genitalia. Transmission is mainly by direct contact with nasal secretions from infected rabbits and may be the greatest when rhinitis induces sneezing and aerosolization of secretions [2]. A syndrome of atrophic rhinitis or degeneration of the nasal turbinates has been associated with toxin-producing strains of *P. multocida* in rabbits [3]. Colonization and disease are influenced by factors related to both the host and the pathogen. Different strains of *P. multocida* have been isolated from rabbits [4]. They are classified by capsular type and serotype; A:12 is the most common in rabbits in the United States, but also A:3 and other A and D serotypes exist [5]. A more severe disease has been associated with A:3 and D strains [6]. Both capsular types D and A have been shown to produce a toxin [7]. Investigation of the biochemical properties of *Pasteurella multocida* is also important for a better understanding of the pathogenesis of this disease [4]. The ability of a rabbit to resist *P. multocida* infection depends, in part, on the health of the exposed mucosa, and probably on rapid production of mucosal antibodies (IgA) that inhibit the growth of the bacteria. High levels of humoral antibodies (IgG) are not associated with elimination of infection but rather with a chronic process [8]. Since most or all rabbits carry *Pasteurella multocida* in the nasal cavity, management measures are aimed at controlling the clinical disease expression [9]. Some antimicrobials are used to control pasteurellosis, although in most cases they are effective only for a short period [10, 11]. Publications on the specific prophylaxis of rabbit pasteurellosis are not numerous. There are some data that attempts to induce immunity and protection using bacterins, potassium thiocyanate extracts or attenuated live bacteria have failed to prevent pasteurellosis over time [12, 13]. However, some manufacturers produce a vaccine against this disaese,

but the antigenic structure of the antigens not always coincides with the antigenic structure of bacteria, which are spread in different areas. For this reason autovaccines should be used [9, 14]. A monovalent vaccine against rabbit pasteurellosis in Lithuania was produced using local *P. multocida* strains. The vaccine was evaluated in experimental and field conditions. The aim of the present work was to select strains for the inactivated pasteurella vaccine and to elaborate effective measures for active immunization of rabbits.

MATERIALS AND METHODS

Bacteriological investigations were done with the aim to isolate *Pasteurella multocida* in Lithuanian rabbit farms. Clinical samples were collected with sterile swabs directly from nasal mucosa. Appropriable means of transportation were used to ensure more objective investigations [15, 16]. Pathological material was also investigated. Investigations were done only from animals with clinical signs typical of pasteurellosis. The biochemical properties of isolated bacteria were tested. Capsular typing was done using antisera against *Pasteurella multocida* K antigen by an indirect haemagglutination test. Supplementary tests of tripaflavine and hialuronidase were done. The pathogenic properties of the isolated strains of *P. multocida* were determined using white mice (BALB/c line) and rabbits of chinchilla breed. *Pasteurella multocida* was considered virulent to laboratory animals in the cases when these animals became ill or died after injection of no less than 10^8 b.c. (bacterial cells) subcutaneosly.

Selection of isolated *P. multocida* strains was done according to their properties for vaccine development. These strains had to be capsulated and highly pathogenic to animals. Their growth, antigenic, biochemical and pathogenic properties had to be stabile for the whole time of observation (no less than 15 passages).

Two strains of *Pasteurella multocida* were used for vaccine development. One of them belonged to capsular type A and the second to capsular type D. *Pasteurella multocida* were cultured on Soya-Trypticase Broth (BBL, USA). Yeast extract was added for the best growth of cultured bacteria. After 18 hours, 0.5% of formaldehyde was added into the medium as an inactivator. After inactivation the bacteria were concentrated by centrifugation and then suspended in saline until their concentration reached 4×10^9 b.c./ml. Each strain was cultured separately. For vaccine production both strains were mixed. The final concentration of the vaccine was 4×10^9 b.c./ml. Thimerosal (Sigma) was used as a preservative. The oil-in-water adjuvant "Emulsigen" (MVP Laboratories, Inc. USA) was used for a better immune response.

Tests for vaccine sterility were done according to Lithuanian standards in force. The safety of the vaccine was tested by rabbit immunization. 2 ml of the vaccine were injected to each rabbit (10 rabbits in a group). Injections were repeated after 10 days at the same dose. The effectiveness of the vaccine was determined using three different doses. There were four groups of weaned off (aged 6 weeks) rabbits, four rabbits in each. The first group of rabbits were immunized with 0.5 ml (2×10^9 b.c.), the second group by 1 ml (4×10^9 b.c.) and the third group by 2 ml of the vaccine 8×10^9 b.c). The vaccination was repeated after 14 days the first vaccination. The rabbits of the fourth group were not vaccinated. Twenty-one days after the second vaccination all rabbits were infected with two virulent *P. multocida* strains (A and D types). The rabbit's survival was criterion of vaccine efficacy and dosage. The percentage of survivors was calculated as a proportion of dead rabbits and survivors after infection with pathogenic strains of *Pasteurella multocida.*

In the course of experiment some immunological indices in rabbit blood were determined. Different tests are used for detection of antibodies to *Pasteurella multocida* antigens [15]. The indirect haemagglutination test was used for this purpose. The count of leukocytes in rabbit blood was determined by counting them in Goriaev's chamber.

Statistical analysis was carried out using the Sigma Plot computer programme (Jandel Scientific, version 1.02a). A value of $p \leq 0.05$ was considered significant.

Clinical trials of the effectiveness of the vaccine were performed using 500 pregnant rabbit females and 5000 of young rabbits. These animals were immunized in three ill-affected rabbit farms. A stock of different breeds was used. Vaccination was carried out for a period of 12 months. All animals were vaccinated, except some individuals that had intense clinical signs. These animals were condemned. Before vaccination, in all farms 15–20% of rabbits (adult and young animals) had clinical signs of pasteurellosis, and the diagnosis was confirmed by bacteriological investigations. 40% of slauthered rabbits had various lesions in the lungs. All rabbits in all farms were immunized with 1 ml of the vaccine. Vaccination was repeated after 14 days. Females were revaccinated in the middle of every third pregnancy. Young rabbits were vaccinated after weaning. Breeding males were revaccinated every 4 months. The frequency of clinical signs of rabbit pasteurellosis was observed before and after vaccination. Bacteriological investigations according to pasteurella isolation were carried out on a regular basis.

RESULTS

On 12 different rabbit farms, from 75 specimens of rabbit nose mucosa and from pathological material

Table 1. **Number of leukocytes in blood sera of rabbits in the course of experiment**

Group of rabbits	Dose of the vaccine	Number of leukocytes before vaccination, 10^9/l	Number of leukocytes after vaccination, 10^9/l
1	2×10^9 b.c.	6.75 ± 1.9	7.50 ± 0.8
2	4×10^9 b.c.	6.85 ± 1.9	7.90 ± 1.2
3	8×10^9 b.c.	7.05 ± 2.5	8.10 ± 1.0

Table 2. **Rate of rabbits' survival after their infection with pathogenic strains of *Pasteurella multocida***

Group of rabbits	Doses of the vaccine	Infection result		Percentage of survived rabbits
		Survived	Died	
1	2×10^9 b.c.	2	3	40
2	4×10^9 b.c.	5	0	100
3	8×10^9 b.c.	5	0	100
Control	Non vaccinated	0	5	–

(lungs, abscess-affected mammary glands) 11 strains of *Pasteurella multocida* were isolated and identified. Alongside *Pasteurella multocida*, also some grampositive and gramnegative bacteria (*Streptococcus* spp. *Staphylococcus* spp. and *E. coli*) were isolated and identified. All isolated *Pasteurella multocida* isolates had typical biochemical properties: they produced oxidase and catalase, fermented carbohydrates, produced indole, reduced nitrates to nitrites, were non-motile and did not grew on MacConkey agar. Five strains grew in M growing form, 4 strains in S and 2 strains in R growing form. Five strains belonged to capsular type A, 3 strains to capsular type D. Three strains were acapsulated. Only four strains were highly virulent to laboratory animals (two strains of capsulotype A and two strains of capsulotype D). For vaccine development, one strain of capsular type A and one strain of capsular type D were selected. Their growing, biochemical and pathogenic properties were stabile throughout the whole period of observation.

The vaccine prepared from two strains of *Pasteurella multocida* (A and D capsular types) was sterile and safe to target animal species. A slight increase of the rabbit's body temperature was observed on the first day after vaccination. A mild transient reaction at the site of injection was observed at the period of investigations independently of the dose of the vaccine.

Before vaccination, no specific antibodies against pathogenic *Pasteurella multocida* were detected in rabbit blood sera (the initial dilution of sera was 1:20).

On the 21st day after the second vaccination, the titres of specific antibodies were determined. They are shown in Figure. One can see that the specific antibody titres against *Pasteurella multocida* were rather high, but the highest mean values were reached when the vaccine dose was no less than 1.0 ml injected subcutaneously. There was no increase of titres in the blood of nonvaccinated rabbits.

The dynamics of the number of leukocytes is shown in Table 1. The number of leukocytes increased in all rabbit groups. Increasing leukocyte counts show that the organism is ready to resist the infection. The highest differences before and after vaccination were observed in the second and third groups. Part of the rabbits infected with the pathogenic *Pasteurella multocida* strains died; the others survived depending on the administered dose of the vaccine (Table 2).

A 100% rabbits' survival was observed when the dose of the vaccine was no less than 1 ml and the vaccine contained no less than 4×10^9 b.c./ml (Table 2). A lower dose (0.5 ml) was insufficient to evoke a sufficient immunity. The difference of results between group 1 and groups 2–3 of rabbits was statistically reliable ($P < 0.05$). All rabbits of the control group died.

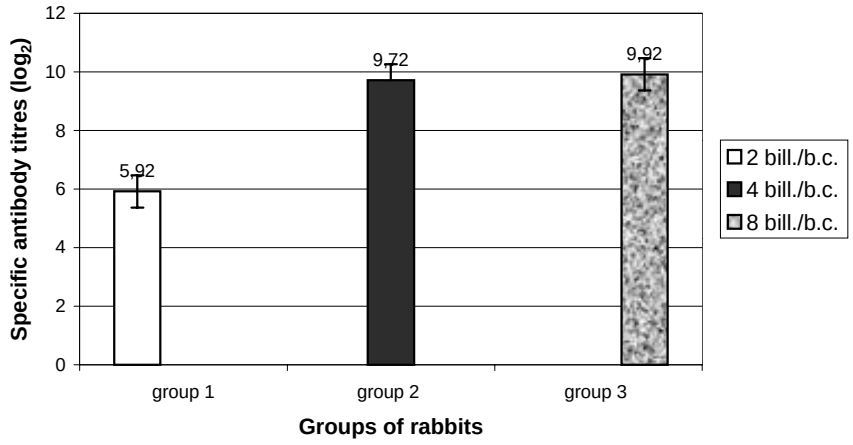

Figure. Specific antibody titres in blood sera of rabbits vaccinated with different amounts of the vaccine

In the rabbit farms, 12 months following the active immunization of adult and young rabbits, the number of rabbits that had clinical signs of pasteurellosis significantly decreased. After 12 months, only 2% of rabbits had sneezing and other disorders of the upper respiratory tract in all farms. Slaughtering revealed only 12% of rabbits to have lung lesions. Bacteriological investigations showed that *Pasteurella multocida* in some cases was present in young and adult animals, but the clinical signs of disease significantly decreased.

DISCUSSION

Pasteurella multocida is one of the most frequent bacteria in rabbit farms. Besides *P. multocida*, some other species of bacteria (staphylococci, streptococci and *E. coli*) were isolated. These and also some other species of bacteria (*Klebsiella pneumoniae, Bordetella bronchiseptica*, *Pseudomonas aeruginosa*) are common pathogens of rabbit respiratory tract [17]. From 75 clinical samples, 11 strains (14.6%) of *Pasteurella multocida* were isolated. 45% of the isolated pasteurella belonged to capsular type A, 27% to capsular type D and 27% were acapsulated. Only 4 strains (36%) of the isolated *Pasteurella multocida* were virulent to laboratory animals. According to the data of some authors, many rabbits are asymptomatic carriers of *Pasteurella multocida* [9]. Data of other authors are contrary: some authors maintain that among various bacteria isolated from healthy rabbits there were no one case of *Pasteurella multocida* [17]. Our results show that rabbits often are carriers of *Pasteurella multocida*, but the pathogenicity of the strains may be different. Therefore, it is very important to determine the pathogenicity of the isolated *Pasteurella multocida*. The results of such determination may be important for disease diagnosis and prophylaxis.

There are some reasons that may impact the frequency of *Pasteurella multocida* isolation. One reason is an affected rabbitry where pasteurellosis acts as a primary disease. In this case all rabbits in a farm may be affected, because these bacteria spread by direct contact, through aerosol, venereal and haematogenous routes. The epidemiology of pasteurellosis also may vary according to the type of breeding conditions (small-scale breeding, laboratory facilities, professional large-scale breedings) [9]. The ratio between virulent and avirulent *Pasteurella multocida* strains also may be different. It depends on many factors such as an individual rabbit farm, a common immune status of the farm, region, time after disease expression in a region, etc. Some rabbit farms in Lithuania are affected by *Pasteurella multocida*. One of the reasons is insufficient control of the breeding stock. Most brood animals were brought from other countries and not always by a legal way. The breed stock infected with *Pasteurella multocida* then spread among farms and almost all biggest farms, particularly those breedding rexes, became *P. multocida* carriers.

A laboratory trial of the vaccine against rabbit pasteurellosis using rabbits as experimental and at the same time as target animals revealed that 100% of vaccinated rabbits survived after *Pasteurella multocida* infection when the vaccine concentration was 4×10^9 b.c./ml by immunization of rabbits with no less than 1 ml of the vaccine. The ratio of both pasteurella capsular types in the vaccine was 1:1 and it appeared to be suitable for vaccine production. Different bacterial strains have not equal immunogenicity, but an adjuvant can help longer conserve the antigen in the body and use less amounts of the antigen for the development of sufficient immunity [18]. Adjuvants of the new generation, such as “oil-in-water”, give no adverse reactions in animals and are safe. Some authors predicate that only vaccines prepared from local strains of *Pasteurella multocida* can be effective against rabbit pasteurellosis [9]. Our trials confirm that a vaccine from local strains may be effective.

The vaccine had no adverse effects on rabbits, except sterile swelling at the site of injection. It is common when animals are vaccinated with inactivated vaccines with oil adjuvants.

There was a statistically significant difference between the first and the other two groups of rabbits in the specific antibody titres, number of leukocytes and survival after experimental infection.

A direct correlation was determined among the specific antibody titres in blood sera, number of leukocytes, and a protective vaccine concentration. Thus, the results of our study show that our vaccine was safe and effective under experimental conditions. According to mean specific antibody titres, leukocyte count and rabbits’ survival after infection it may be defined that the optimal dose of the vaccine for rabbits is 1 ml.

The results after immunization in pasteurella-affected rabbitries were good. In spite of that some animals remained *P. multocida* carriers, clinical signs of pasteurellosis in the farms significantly decreased. All rabbits in the farms were vaccinated regardless of their illness, because it is difficult to separate healthy animals from those sick, especially when pasteurellosis is caused by low toxigenicity *P. multocida* and the clinical signs are slightly expressed. It is normal that some rabbits remain *Pasteurella multocida* carriers, besause *Pasteurella multocida* may survive in some immune cells (macrophages) and may be resistant to neutrophil phagocytosis [19]. In this case, it is more important that the clinical signs of pasteurellosis significantly decreased and the rabbitries were saved from full dissolution. The best way to prevent rabbits from pasteurellosis is a good barrier tech-

nique and formation of a colony with a SPF stock. Statistically reliable results were obtained according to clinical signs and pathological lesions before and after vaccination. Trials showed that clinical signs after vaccination in the rabbit farms reduced 7 to 10 times (from 15–20% to 2 %). Lung lesions decreased more than 3 times (40% before vaccination and 12% after vaccination). The obtained data allow to conclude that vaccination may be used as one of the most effective measures against rabbit pasteurellosis.

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INAKTYVUOTOS PASTERELIØ VAKCINOS TRIUÐIAMS SUKÛRIMAS IR IÐBANDYMAS

Santrauka

Tyrimø tikslas – atrinkti *Pasteurella multocida* rûðies bakterijø padermes, atitinkanèias biologiniams preparatams keliamus reikalavimus, ir pagaminti inaktyvuotà pastereliø vakcinà triuðiams, laboratoriniais ir klinikiniais tyrimais ávertinti pagamintos vakcinos saugumà ir efektyvumà.

Bakteriologiniai tyrimai atlikti ið ávairiø respublikos apskrièiø pristaèius serganèiø ar nugaiðusiø triuðiø patologinæ ir klinikinæ medþiagà á LVA VI Mikrobiologijos ir maisto saugos skyriaus laboratorijà. Ið 75 nugaiðusiø ar sirgusiø triuðiø vidaus organø iðskirta 11 pastereliø padermiø (14,6%) ir iðtirtos jø kultûrinës, patogeninës savybës, toksigeniðkumas. Iðskirtø pastereliø serologiniai tipai charakterizuoti pagal kapsuliná (K) antigenà netiesioginës hemagliutinacijos reakcijos bûdu. Penkios padermës (45%) priklausë kapsuliniam tipui A, trys padermës (27%) – kapsuliniam tipui D. Trys padermës (27%) kapsuliø neturëjo. Á vakcinos sudëtá átraukta po vienà abiejø nustatytø kapsuliniø tipø pastereliø padermæ. Kaip adjuvantas panaudotas komercinis adjuvantas emulsigenas (Emulsigen, MVP laboratories, Inc., JAV).

Laboratoriniai tyrimø rezultatai rodo, kad visi vakcinuoti triuðiai ágijo imunitetà prieð pastereliozæ, kai bakterijø skaièius vakcinoje buvo ne maþesnis nei 4×10^9 viename mililitre, vakcinuojant triušius doze, ne maþesne nei 1,0 ml. Abiejø kapsuliniø tipø pastereliø santykis vakcinoje buvo vienodas, ir eksperimentai patvirtino, kad toks santykis uþtikrino vakcinuotø triuðiø apsaugà nuo abiejø kapsuliniø tipø patogeniniø padermiø.

Vakcina buvo saugi ir neturëjo ðalutinio poveikio, iðskyrus laikinà, nedidelá patinimà injekcijos vietoje. Iðbandant pagamintà vakcinà klinikinëmis sàlygomis (serganèiø pastereliozе triuðiø fermose) gauti patenkinami rezultatai. Po imunizacijos triuðiø fermose nuo 8 iki 10 kartø sumaþëjo pastereliozei bûdingø klinikiniø simptomø kiekis, o patologiniai-anatominiai plauèiø pakitimai – daugiau nei 3 kartus. Ávertinus gautus rezultatus galima teigti, kad triuðiø vakcinacija gali bûti viena ið svarbiausiø priemoniø apsaugant triuðius nuo pastereliozës.