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EFFICACY OF AN AUTOGENOUS VACCINE AGAINST HIGHLY VIRULENT *STAPHYLOCOCCUS AUREUS* INFECTION IN RABBITS

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Abstract: The efficacy of an autogenous vaccine consisting of a whole cell suspension of formalin killed bacteria in sterile buffered saline against *Staphylococcus aureus* infections was determined, using a well-established rabbit skin infection model. Thirteen 8 wk old rabbits were vaccinated twice subcutaneously with a 2 wk interval while 10 rabbits were injected twice with formalised PBS (sterile phosphate buffered saline). Two weeks after the last injection, 10 vaccinated and all PBS-injected rabbits were inoculated intradermally with 10^8 cfu of a *S. aureus* strain (KH 171)which had been shown to be highly virulent for rabbits. Three vaccinated animals served as negative controls and were intradermally injected with sterile buffered saline. All rabbits were examined daily for the development of skin lesions until 14 d after the experimental infection when all rabbits were euthanised. All animals experimentally infected with *S. aureus* developed skin abscesses within 24 h post-inoculation, but in the vaccinated group the maximum abscess diameter was significantly lower than in the non-vaccinated group (P=0.048). This difference between autovaccinated and non-vaccinated groups increased over time (P<0.001). These results indicate that vaccination with an inactivated whole cell bacterin may be useful for control of staphylococcosis in rabbits but does not prevent abscess formation in animals inoculated with a high dose of a highly virulent *S. aureus* strain.

Key Words: *Staphylococcus aureus*, virulence, infection, rabbit, skin, autovaccine.

INTRODUCTION

Staphylococcus aureus causes many problems in rabbits such as subcutaneous abscesses, mastitis, pododermatitis, and can occasionally evolve to septicaemia (Corpa *et al.,* 2009). It is commonly accepted that these symptoms occur after infection of a wound with *S. aureus*. At rabbit flock level, two types of infection can be distinguished. In the first type, caused by low virulence (LV) strains, only a few number of animals develop symptoms and the economic importance therefore remains low. In the case of the second type of infection, caused by high virulence (HV) strains, the disease spreads throughout the entire flock affecting general health, growth and reproduction parameters. This leads to chronically poor production results and increased slaughterhouse condemnations. The use of antibiotics in feed or

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drinking water or topically applied does not offer an effective therapy (Okerman *et al*., 1984; Hermans *et al*., 2003) and often all animals have to be culled due to the high economic losses. In rabbit farming, vaccination would thus be a very valuable alternative to control the disease. In general, attempts to develop a vaccine against *S. aureus* encounter many difficulties (Lee, 1996; Projan *et al*., 2006). Vaccines that have been tested in animals include whole-cell vaccines based on live and killed *S. aureus* bacteria, capsular vaccines and protein vaccines such as alpha-toxoid vaccine. Rabbits have been used as a model for testing the efficacy of bovine *S. aureus* vaccines. In a rabbit mastitis model, some degree of protection against challenge, toxin spread and subsequent death was found after vaccination with purified alpha- and beta-toxins from bovine *S. aureus* strains, but not against abscess formation (Adlam *et al*., 1977). Bovine mastitis vaccines, based on whole bacterial cells, crude toxoid (α- and β-haemolysins) or combinations of both induced variable protection in a rabbit skin infection model depending on the strain combination used, the amount of toxoid included in the vaccine and the number of vaccine injections (Cameron *et al*., 1979). Hinton (1977) reported that therapeutic administration of an autogenous *S. aureus* vaccine resulted in a temporary clinical improvement of conjunctivitis in rabbits. To our knowledge, there are no other reports on the efficacy of vaccines specifically designed for use in rabbits.

In the field, autogenous vaccines are sometimes used in an attempt to control the problems with HV *S. aureus* in rabbits at flock level, with variable success rates. Results of vaccination in the field may be influenced by several interfering factors including concurrent diseases and management practices. Therefore, the aim of this study was to evaluate the effect of an autogenous bacterin in a well-standardised rabbit skin infection model (Meulemans *et al*., 2007).

MATERIAL AND METHODS

Experimental animals

The animal experiments were carried out after approval from the ethical committee of the Faculty of Veterinary Medicine of Ghent University. Twenty-three 8 wk albino hybrid rabbits (ILVO, Melle, Belgium), weighing 2.0-2.5 kg and from either sex were kept individually in polyethylene benches and received food and water *ad libitum*. Before the onset of the experiment, rectal temperature was measured and the following body sites were sampled for bacteriological examination as described by Hermans *et al*. (1999): the nares, auditory canal, interdigital skin, medial skin of the right foreleg, axillar and inguinal skin region, skin around the nipples, perineum and vagina or preputium. The samples were screened for the presence of HV *S. aureus* strains.

Preparation of the challenge inoculum

In all experiments, the highly virulent (HV) *S. aureus* isolate KH 171 (Hermans *et al*., 1999) was used. This strain belongs to the biotype mixed CV-C, is sensitive to the phages of phage group II (3A, 3C, 55 and 71) displays spa type t645, MLST type ST121 and therefore belongs to the widely disseminated clone that has previously been described as highly virulent (Vancraeynest *et al*., 2006; 2007). Originally, the strain was isolated in a Belgian rabbitry experiencing severe problems with mastitis, pododermatitis, pustular dermatitis and subcutaneous abscesses. Its virulence has been demonstrated in a rabbit intranasal colonisation experiment (Hermans *et al*., 2000) and in a rabbit skin infection model (Meulemans *et al*., 2007).

The isolate was grown for 24 h at 37°C on Columbia agar (Gibco, Paisley, Scotland) containing 5% ovine blood (blood agar) in a 5% CO_2-enriched environment. After checking the strain for purity, 10 colonies were transferred into brain heart infusion (BHI) (Oxoid, Basingstoke, England) broth for 24 h at 37°C whilst shaking. The number of colony forming units (cfu) was determined by inoculation of 10-fold

dilutions on blood agar and the suspension was stored overnight (O/N) at 4°C in phosphate buffered saline (PBS). Prior to the experiment, the suspension was centrifuged at 1500 rpm and the pellet adjusted to an appropriate concentration of 10^9 cfu/mL PBS (10^8 cfu/0.1 mL PBS).

Preparation of the autogenous vaccine

S. aureus strain KH 171 was grown for 24 h at 37°C on blood agar in a 5% CO_2-enriched environment. After checking the purity of the strain, 5 colonies were transferred into 200 mL of sterile Columbia broth (Difco, Becton Dickinson and Company, Sparks, USA) and incubated for 24 h at 37°C whilst shaking. After checking its purity and confirmation of the identity of the strain, 1 mL of formaldehyde 36% (VWR International, Fontenay-sous-Bois, France) was added and the broth was incubated O/N at 37°C.

The following day, the sterility of the suspension was checked by inoculation onto a blood agar plate. The broth was then centrifuged at 5000 rpm for 30 min at room temperature. The supernatant was discarded and the pellet was resuspended in 100 mL of sterile PBS with 0.5% formaldehyde and the concentration of the formalised staphylococcal suspension was assessed using a McFarland nephelometer. The suspension was again incubated at 37°C O/N and after dispensing it into sterile glass vaccine bottles, sterility was confirmed by direct inoculation of a loopful of the vaccine suspension onto blood, Sabouraud (Oxoid, Basingstoke, England) and PAM agar (Difco, Becton Dickinson and Company, Sparks, USA) and incubating for 7 d at 37°C. Additionally, 1 mL of the formalised suspension was added to 10 mL of brain-heart infusion (BHI), thioglycollate, Sabouraud (Oxoid, Basingstoke, England) and PPLO (Difco, Becton Dickinson and Company, Sparks, USA) broth at 37°C. Inoculated broths were examined daily visually for 2 wk for the absence of bacterial growth and their sterility was confirmed by inoculation on the previously mentioned agars and incubating for 7 d at 37°C.

Experimental design

The rabbits were randomly allocated to 2 groups of 10 animals (group A=vaccinated; group B=non-vaccinated) while 3 animals were kept as negative controls. The rabbits of group A and the 3 negative control animals were vaccinated twice subcutaneously with 0.5 mL of the bacterin with an interval of 2 wk. At the same time, the rabbits of group B were injected twice with 0.5 mL of formalised PBS. Two weeks after the second injection, rabbits of group A and B were anaesthetised with isoflurane (IsoFlo®, Abbott Laboratories Ltd., Queensborough, England) by mask induction. As previously described by Meulemans *et al*. (2007), the fur of the right flank was shaved with electric clippers (Contura, Wella, Germany) and disinfected thoroughly with 70% ethanol (Disinfectol®, Chem-Lab NV, Zedelgem, Belgium) for 5 min. After evaporation of the ethanol, a tattoo pin (letter "O"; width×length=5×7mm) was pressed into the skin, producing a 0.5 mm deep "O"-shaped dermal skin lesion. The lesion was then inoculated with 10^8 cfu of the KH171 *S. aureus* strain in 0.1 mL PBS. In the negative control animals, the same procedure was followed but the skin lesions were sham inoculated with 0.1 mL sterile PBS. After the inoculum was air-dried, the rabbits were allowed to recover from the anaesthesia.

Rabbits were inspected daily for the development of macroscopic skin lesions and their rectal temperatures were measured. Skin lesions were measured by means of a vernier calliper. The person measuring and scoring the lesions was unaware of the treatment assignment (blinding). At 14 d post-inoculation (pi) the animals of groups A and B were euthanised and swab samples of the nares and flanks (abscesses) were collected for bacteriological analysis, as were skin biopsies for histopathological examination. All animals were then subjected to a full necropsy to inspect internal organs for possible pathological changes.

Bacteriological examination

All samples were inoculated onto blood agar. Samples of the nares and perineum were additionally cultured on modified Baird-Parker agar (Devriese, 1981). After 24 h incubation at 37°C, *S. aureus* colonies were identified and classified into biotypes as described by Devriese (1984). Phage typing of the isolated *S. aureus* colonies was performed at the Scientific Institute of Public Health (Brussels, Belgium) using bacteriophages of the international typing set for human *S. aureus* strains according to Parker (1962).

Histopathological examination

Representative skin biopsies of the right flank with underlying muscle and biopsies of other encountered lesions were fixed in 10% buffered formalin, paraffin embedded and cut into 5 µm slices. After conventional haematoxylin-eosin (HE) staining, tissue sections were evaluated using light microscopy. Histopathological lesions were classified according to the following parameters: crust formation, epidermal hyperplasia, degree of necrosis and type of inflammation in different dermal parts (superficial, mid and deep dermis) and the subcutis.

Statistical analysis

The LOCF (last observation carried forward) procedure was used for animals euthanised before the end of the study period, assigning the value of the last observation for the remaining time points. Two different parameters were compared statistically. Firstly, the maximum abscess diameter reached after inoculation was compared for the challenged animals from both groups. This parameter is a reflection of the severity of the inflammatory reaction and will have an impact on the possibility of healing (Cotran *et al*., 1999). This statistical comparison was performed by means of a one-sided Wilcoxon rank sum test. Secondly, in order to evaluate the development of the abscess diameter over time, a mixed model analysis was performed with animal as random effect, and time, autovaccine and their interaction as fixed categorical effects. Within the mixed model, control and vaccinated groups were compared per day using Bonferroni's multiple comparisons technique with a significance level of 0.0036 (i.e. 0.05/14, as the abscess measurements on different days are not independent from each other).

RESULTS

Clinical examination

Four rabbits showed clinical signs of pain and loss of appetite and were therefore euthanised at 8 d pi. The rest of the animals remained alert and showed normal appetite and grooming activities throughout the whole experiment. Rectal temperatures stayed within the normal range (Harcourt-Brown, 2002) and varied between 37.8°C and 39.6°C.

Macroscopic evaluation of lesions

The 3 animals from the unchallenged vaccinated group (negative controls) did not develop any lesions. The animals from both other groups developed an abscess on the right flank within 24 h pi, with a diameter varying from 8 to 40 mm. The evolution of the mean abscess diameters in both the autovaccinated (group A) and non-vaccinated (group B) rabbits during the 2 wk observation period after challenge with the HV *S. aureus* strain is represented in Figure 1.

At 8 d pi, 1 rabbit from group A and 3 rabbits from group B showing clinical signs of pain and loss of appetite, were euthanised for ethical reasons. Those animals had abscesses with a minimum diameter of at least 55 mm for 3 subsequent days.

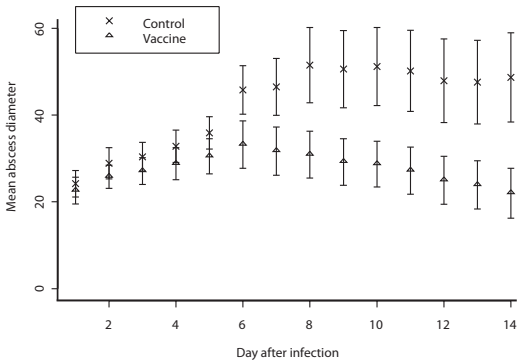

Figure 1: Evolution of the mean abscess diameters (in mm) in the autovaccinated group A (Δ) and the non-vaccinated group B (×) during two weeks after challenge with a HV *S. aureus* strain.

In the challenged and vaccinated group A, all animals developed abscesses on the right flank of at least 20 mm within the 1st wk of the study, with diameters ranging from 20-65 mm. From then onwards, all abscesses of the 9 surviving animals started to regress. The maximal abscess diameter was reached within the first 48 h pi for 2 out of 10 animals. The rest of the group reached its maximal abscess size 5 to 7 d after infection. At the end of the experiment, lesion diameters varied between 4 and 38 mm, with 6 of them measuring less than 20 mm.

In the non-vaccinated group B, all animals developed abscesses of at least 18 mm. Eight of them reached their maximum diameter at 5 to 8 d after inoculation with diameters ranging from 18-90 mm. In one rabbit, maximal nodule size was noted at the last day of the experiment. Another rabbit developed an abscess of 30 mm after 24 h that started to regress after 1 d to enlarge again to 28 mm at 4 d. After a second regression to 14 mm at 12 d, it enlarged again to 24 mm on the last day of the experiment. At the end of the experiment lesion diameters from 9 to 77 mm were noted in group B.

In 6 out of 10 animals from group A and 7 out of 10 animals from group B, a small abscess (1-3 mm) appeared on the lower and/or upper lips from 7 or 8 d pi onwards.

Bacteriological examination

From the body samples taken before the experiment was started, no HV *S. aureus* was isolated.

At the end of the experiment, 3 rabbits each in both groups A and B were positive for HV *S. aureus* in the nose. In the vaccinated group A, 9 rabbits were positive on the right flank compared to 10 rabbits in group B. No changes were noted in the biotype or phage type of the isolates obtained at the end of the experiment, compared to the inoculated strain.

Abscess diameter

Firstly, the maximum abscess diameter reached after inoculation was compared for the animals of both groups. The autovaccinated animals had a significantly (P=0.0477) lower maximum abscess diameter than the control animals (Figure 1).

Secondly, the development of the abscess diameter during the course of the experiment was evaluated. The abscess diameter changed significantly over time (P<0.001) and in a different way in the 2 groups (significant interaction between group and time; P<0.001): until 5 d, the differences between the 2 groups were small, but thereafter the differences increased mainly due to the fact that the abscess diameter slightly decreased in the autovaccinated group whereas it continued to increase in the control group. The largest difference in abscess diameter between the challenged vaccinated and non-vaccinated group was found on the final day of the experiment, where it just failed to be declared statistically significant as the P value was slightly higher than 0.0036 (P=0.005). Globally, there was only a tendency towards a smaller average abscess diameter in the vaccinated group (P=0.077).

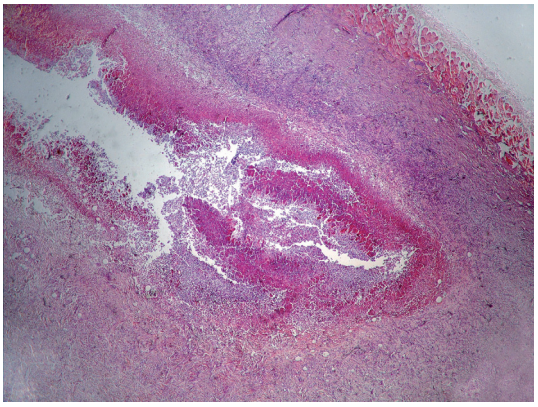

Figure 2: Histopathological changes (haematoxylin-eosin stained sections, light microscope, 25×) in the skin biopsy of a non-vaccinated rabbit euthanised at 8 d post-inoculation, showing an extensive area of dermal necrosis with a massive infiltration of degranulating polymorphonuclear leukocytes and some monocytes.

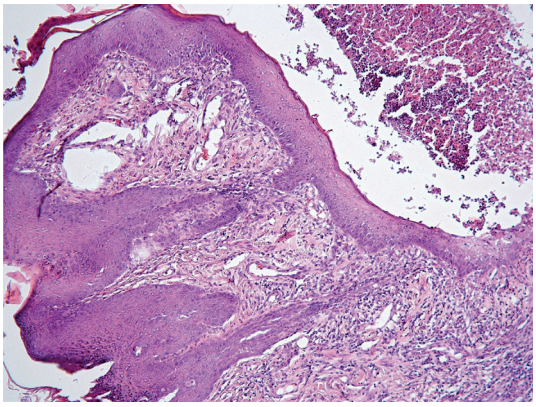

Figure 3: Histopathological changes (haematoxylin-eosin stained sections, light microscope, 50×) in the skin biopsy of an autovaccinated rabbit (group A) at 2 wk after infection, showing epidermal hyperplasia, mild subepidermal oedema and a mild to moderate superficial suppurative perivasculitis. In the mid to deep dermis there was evidence of fibrosis, follicular atrophy and mild to moderate multifocal perivasculitis with predominant infiltration of mononuclear cells and some polymorphonuclear leukocytes.

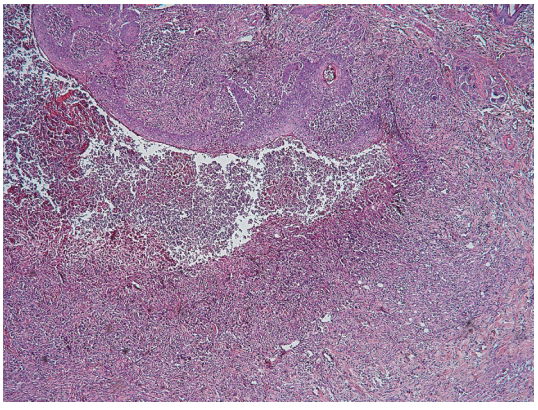

Figure 4: Histopathological changes (haematoxylin-eosin stained sections, light microscope, 50×) in the skin biopsy of a non-vaccinated rabbit (group B) 2 wk after infection, showing moderate to marked epidermal hyperplasia with intercellular oedema and transmigration of PMN. The dermis showed oedema and a moderate perivasculitis with marked infiltration of polymorphonuclear leukocytes and some mononuclear cells.

Histopathological examination

The skin of the 4 rabbits euthanised at 8 d showed an extensive area of necrosis in the mid to deep dermis with a massive infiltration of degranulating polymorphonuclear leukocytes (PMN) and some monocytes. There was also evidence of collagenolysis and myolysis. The histopathological changes in the skin of a non-vaccinated rabbit at 8 d are presented in Figure 2.

In group A, skin biopsies of the animals euthanised at the end of the experiment showed moderate to marked epidermal hyperplasia, mild subepidermal oedema with occasional transmigration of PMN and mild to moderate superficial suppurative perivasculitis. In the mid to deep dermis there was evidence of fibrosis, follicular atrophy and mild to moderate multifocal perivasculitis with predominant infiltration of mononuclear cells (monocytes, lymphocytes and plasma cells) and some PMN. In the muscular layer, there was focal infiltration of monocytes and lymphocytes. These

histological findings are presented in Figure 3. One animal showed a well-demarcated dermal infiltration of necrotic PMN surrounded by proliferating fibroblasts and some multinuclear giant cells.

All animals in group B showed moderate to marked epidermal hyperplasia with intercellular oedema and transmigration of PMN. In 2 cases there was epidermal necrosis and crust formation. The dermis showed oedema and a moderate mid to deep perivasculitis with marked infiltration of PMN and some mononuclear cells. The histopathological changes in the skin of a non-vaccinated animal at the end of the experiment are shown in Figure 4.

No pathological changes were found in the internal organs of the necropsied rabbits.

DISCUSSION

Although the administration of autogenous bacterin had a significant effect on the maximum abscess diameter in rabbits inoculated with a high dose of a highly virulent *S. aureus* strain, it was not able to prevent abscess formation or the spread of the bacteria towards other body sites, such as the nose and lips. Such high inoculation doses trigger an acute inflammatory reaction with the formation of large abscesses filled with pus. Once this stage is reached, the retraction of the abscess takes a considerable amount of time. This is reflected in the histological findings, where a marked dermal fibrosis was seen at the end of the experiment. It is however very unlikely that a naturally occurring wound under field conditions would be infected with such a high number of staphylococcal bacteria (10^8 cfu). Repeating the experiment with a lower inoculation dose would probably lead to better efficacy of the autovaccine. In literature, skin infection studies using doses of 10^6 cfu *S. aureus* bacteria have been reported (Marples and Kligman, 1975; Kraft *et al*., 1986). Preliminary (unpublished) studies however have demonstrated that it is very difficult to obtain standardised results with lower inoculation doses. Inoculation of rabbit skin with 10^6 cfu *S. aureus* resulted in abscesses with variable lesion sizes that disappeared within 1 wk in some of the animals.

When comparing the abscess size in both groups over time: until 5 d pi, the differences between groups were small. From then on, the abscess diameter slightly decreased in the autovaccinated group compared to the non-vaccinated group, where it continued increasing. These findings suggest that in the autovaccinated animals the resolution time of the abscess is reduced. The largest difference between both groups was found at the final day of the experiment, suggesting a positive ongoing effect in the autovaccinated animals. It may therefore be useful to perform trials with longer observation periods, as it is possible that the diameter in vaccinated animals will continue to decrease and healing of the abscesses will occur. Field studies on autogenous bacterin therapy against *S. aureus* in cattle (Hoedemaker *et al*., 2001) and *S. pseudintermedius* (Bannoehr *et al*., 2007) in dogs (Curtis *et al*., 2006) have followed up patients for more than 2 mo. Furthermore, field vaccination trials include much larger animal groups compared to our study. The use of larger groups could minimise possible individual effects of the administered autovaccine on the animal. If some of the rabbits had to be euthanised before the end of the experiment, there would still be a large group of animals left to follow over time. However, the purpose of this study was to test the autovaccine in the previously developed skin infection model with a sample size allowing the detection of statistically significant differences at a P level of 0.05 between groups A and B.

In this experiment, animals were vaccinated twice with a 2 wk interval and challenged 2 wk after the second injection. By adapting the vaccination schedule or applying a longer interval between vaccination and infection, the effect of the administered autovaccine could possibly be enhanced. A positive effect of vaccination against bovine *S. aureus* was seen in some studies using vaccination schedules differing from the schedule used in this study. In a rabbit model where the efficacy of several bovine *S. aureus* vaccines was evaluated, the immunisation schedule consisted of 3 subsequent injections with a 10 d

interval with a vaccine composed of somatic cell antigens and toxoid. After the challenge, which was performed 3 mo after immunisation, the lesions were significantly less severe than in the non-vaccinated controls. Depending on the virulence of the challenge strain, this immunisation still had an effect in some animals 6 mo after vaccination (Cameron *et al*., 1979). In a therapeutic vaccination trial of staphylococcal conjunctivitis in rabbits, Hinton (1977) noted a clinical improvement when using several eight week courses with weekly subcutaneous bacterin administration. Only a temporary improvement was achieved, however.

In the study presented here, the vaccine consisted only of washed formalin-killed bacteria. The addition of the culture supernatant, containing staphylococcal enzymes and toxins might enhance the efficacy of the vaccine. Several studies on vaccine development for the prevention of bovine staphylococcal mastitis focus on the use of vaccines containing inactivated *S. aureus* supplemented with toxoid, capsule or capsular antigens. Those supplements to the bacterin are considered to enhance its efficacy. A vaccine consisting of inactivated, highly encapsulated *S. aureus* cells, inactivated unencapsulated *S. aureus* and crude extract of *S. aureus* exopolysaccharides was tested in heifers and dairy cows in field trials (Calzolari *et al*., 1997; Giraudo *et al*., 1997). This vaccine was able to reduce intramammary infection rates but did not offer full protection. A study performed on subclinical mastitis in lactating cows, testing the effectiveness of an autogenous bacterin with added crude toxoid components, revealed similar findings (Hwang *et al*., 2000).

No adjuvant was used in this trial. Although previous experiments showed no difference in the degree of protection provided by vaccines with or without adjuvants (Cameron *et al*., 1979), the use of an appropriate adjuvant, suitable for rabbits, might enhance the effect of autovaccine administration.

In conclusion, a positive effect of the administered autogenous bacterin on the maximum abscess diameter have been observed. The use of an autovaccine in the present study was however not able to prevent abscess formation in rabbits infected with high doses of *S. aureus* bacteria. To fully investigate the effect of the use of an autovaccine, further studies with an emphasis on adapting the vaccination schedule or modifying the vaccine by adding *S. aureus* culture supernatant or a suitable adjuvant need to be carried out.

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