

# POSSIBLE PROTECTIVE EFFECT OF AN AUTOVACCINE AGAINST HIGH VIRULENCE *STAPHYLOCOCCUS AUREUS* IN A RABBIT SKIN INFECTION MODEL

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## ABSTRACT

The spread of a highly virulent (HV) *Staphylococcus aureus* strain into a rabbit flock leads to a high percentage of rabbits suffering from subcutaneous abscesses, pododermatitis, mastitis, infertility and death with chronic poor production results for the rabbitry. In practice often all animals have to be culled and the rabbitry has to restart with a new flock. Some rabbit farmers however prefer to use an autovaccine, manufactured from the specific HV *S. aureus* strain circulating in their rabbitry, in an attempt to control the infection at flock level. Since the efficacy of such an autovaccine has not been scientifically proven, a double blinded experiment was designed to study this effect in an *in vivo* rabbit skin infection model under standardized experimental conditions. For this purpose, a culture of the HV *S. aureus* strain KH 171 was used to produce an autogenous bacterin based on a whole cell suspension of formalin killed bacteria in sterile buffered saline. On day one and fourteen of the experiment, ten twelve-week-old rabbits (group A) received subcutaneous injections with the prepared autovaccine while ten rabbits of group B were injected twice with sterile buffered saline. Two weeks after these last injections, the rabbits were briefly anaesthetised and after shaving and disinfection of the flank, an "O"-shaped tattoo pin was pressed into the skin. This dermal lesion was then inoculated with 0.1 ml of a suspension containing  $10^8$  cfu of the HV *S. aureus* strain and after air-drying of the inoculum, the animals were allowed to recover. Three other animals served as negative controls. During a period of two weeks after infection, the development of skin abscesses was examined and skin lesions were measured daily. Thereafter, all rabbits were euthanized. Inoculation of the skin with the typical HV strain resulted in skin abscess formation in both groups within 24 hours post-inoculation. Statistical analysis showed that there was a significant effect of the autovaccine on the maximum observed abscess diameter for each animal. Secondly, the area under the abscess diameter curve tended to be smaller for the vaccinated group. With repeated measures analysis, there was also only a tendency for the main effect of the autovaccine to be lower. Thus, a positive effect might be attributed to the administration of the bacterin. However, with the vaccination schedule applied in this study, the use of an autovaccine was not able to prevent abscess formation in infected rabbits.

**Key words:** Rabbit, *Staphylococcus aureus*, Autovaccine, Skin.

## INTRODUCTION

*Staphylococcus aureus* causes many problems in rabbits such as subcutaneous abscesses, mastitis, pododermatitis and can occasionally evolve to septicemia. It is commonly accepted that these symptoms occur after the 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 animals develop symptoms and the economic importance therefore remains low. In the second infection, caused by high virulence (HV) strains, the disease spreads throughout the entire flock affecting growth and reproduction parameters. This leads to chronic poor production results and increased slaughterhouse condemnations. The use of antibiotics as feed or drinking water medication

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, it would thus be desirable to have a better method to control the disease. Vaccination could offer a solution. In general, attempts to develop a vaccine against *S. aureus* provide many difficulties (Lee, 1996). Vaccines that have been tested include whole cell vaccines based on live and killed *S. aureus* bacteria, capsular vaccines and protein vaccines such as alpha-toxoid vaccine. Many researches in vaccine development have been done in the field of bovine staphylococcal mastitis in heifers and dairy cows using inactivated, highly encapsulated *S. aureus* cells, crude extract of *S. aureus* exopolysaccharides and inactivated unencapsulated *S. aureus* (Calzolari *et al.*, 1997; Giraudo *et al.*, 1997). The vaccines were able to reduce intramammary infection rates but did not offer full protection. A study performed in lactating cows testing the effectiveness of an autogenous toxoid bacterin on subclinical mastitis revealed similar findings (Hwang *et al.*, 2000). Some reports can be found on rabbit staphylococcal vaccine studies. The rabbit is then however used as a cheaper and easier to handle alternative for bovine mastitis models. Mostly, specific vaccines towards bovine *S. aureus* strains are tested in rabbit skin infection models (Adlam *et al.*, 1977; Cameron *et al.*, 1979) evaluating the degree of abscess formation. Similarly, avirulent mutant strains have been constructed in the assessment of the degree of protection against challenge with homologous or heterologous *S. aureus* strains (Reinoso *et al.*, 2002). No reports can be found on vaccines developed specifically against rabbit HV or LV strains. In practice however, some rabbit farmers prefer to sacrifice a few animals for necropsy to isolate the highly virulent *S. aureus* strain circulating in their rabbitry in view of the preparation of an autovaccine, in an attempt to control the infection at flock level. The autovaccine is then composed of formalin-killed bacteria resuspended in sterile buffered saline.

Since the efficacy of such an autovaccine against a specific highly virulent *S. aureus* strain has not been scientifically proven, it was the aim of this study to evaluate this effect in an *in vivo* rabbit skin infection model under standardized experimental conditions.

## MATERIALS AND METHODS

### Experimental animals

Twenty-three eight-week-old albino hybrid rabbits (ILVO, Melle, Belgium), weighing 2.0–2.5 kg and from either sex were housed individually throughout the experiment and received food and water *ad libitum*. Before the onset of the experiment, from all animals the following body sites were sampled for bacteriological examination as described by Hermans *et al.* (1999): the nares, the auditory canal, the interdigital skin, the skin of the medial right foreleg, the axillar and inguinal skin region, the skin around the nipples, the perineum and the vagina or preputium. The samples were screened for the presence of HV *S. aureus* strains.

### Bacterial inoculum

In this study, the *S. aureus* strain used was the highly virulent (HV) isolate KH 171 (Hermans *et al.*, 1999) belonging to the biotype mixed CV-C and sensitive to the phages of phage group II (3A, 3C, 55 and 71). The strain KH 171 originated from a Belgian rabbitry experiencing severe problems with mastitis, pododermatitis, pustular dermatitis and subcutaneous abscesses and already demonstrated its virulence in a rabbit intranasal colonisation experiment (Hermans *et al.*, 2000a) 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 in a 5% CO<sub>2</sub>-enriched environment. After checking the strain for purity, ten colonies were transferred into brain heart infusion (BHI) (Oxoid, Basingstoke, England) broth for 24 hours and incubated at 37°C whilst shaking. The number of colony forming units (cfu) after growth was determined by inoculation of 10-fold dilutions on blood agar. Bacterial suspensions were stored overnight at 4°C in PBS and prior to the experiment, the suspensions were centrifuged and the pellets adjusted to an appropriate concentration of 10<sup>9</sup> cfu/ml PBS.

### Autovaccine preparation

For the preparation of the autovaccine, the HV *S. aureus* strain KH 171 was grown for 24 h at 37°C in a 5% CO<sub>2</sub>-enriched environment on Columbia agar containing 5% ovine blood and after purity checking, five colonies were transferred into 200 ml of sterile Columbia broth (Difco, Becton Dickinson and Company, Sparks, USA) for 24 h at 37°C whilst shaking. After purity checking and confirmation of the identity of the strain, 1 ml of formaldehyde 36% was added and the broth was incubated overnight at 37°C. The following day sterility of the suspension was checked by inoculating it onto a blood agar plate. The broth was then centrifuged at 5000 rpm for 30 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 100 ml of sterile phosphate buffered saline (PBS) with 0.5 ml formaldehyde 36% and the concentration of the formalized staphylococcal suspension was assessed using a McFarland nephelometer. The suspension was again incubated at 37°C overnight and after dispensing it into sterile glass vaccine bottles, its content was examined daily during two weeks for the evidence of bacterial growth in BHI, thioglycollate, Sabouraud (Oxoid, Basingstoke, England) and PPLO broth (Difco, Becton Dickinson and Company, Sparks, USA). Sterility of these inoculated broths was confirmed after two weeks by inoculating a loopful onto blood agar, Sabouraud agar (Oxoid, Basingstoke, England) and PAM (Difco, Becton Dickinson and Company, Sparks, USA) and incubating overnight.

### Experimental design

The rabbits were randomly divided into two groups of ten animals and three animals were kept as negative controls. The study was designed as such that the person measuring and scoring the lesions could remain blinded and unbiased. The rabbits of group A and the three negative control animals were administered 0.5 ml of the bacterin solution subcutaneously with two weeks of interval. The rabbits of group B received 0.5 ml of formalized PBS. Two weeks after the last 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 hair of the right flank was shaved with electric clippers (Arco, Moser, Germany) and disinfected thoroughly with 70% ethanol (Disinfectol®, Chem-Lab NV, Zedelgem, Belgium) during five minutes. After evaporation of the ethanol, a tattoo pin (5 x 7 mm) was pressed into the skin producing a 0.5 mm deep “O”-shaped dermal skin lesion. The lesion was then inoculated with 10<sup>8</sup> cfu of the KH171 *S. aureus* strain in 0.1 ml PBS. The skin lesions of the negative control animals were sham inoculated with 0.1 ml sterile PBS. After air-drying of the inoculum, the rabbits were allowed to recover from the anaesthesia. Rabbits were inspected daily for the development of macroscopic skin lesions and the rectal temperature was measured. Skin lesions were measured by means of a vernier calliper. At 14 days post-inoculation the animals of group A and B were euthanized, except for animals that had already been euthanized earlier during the experiment for ethical reasons and swab samples were collected for bacteriological analysis after incision of the abscess with a sterile scalpel blade.

### Statistical analysis

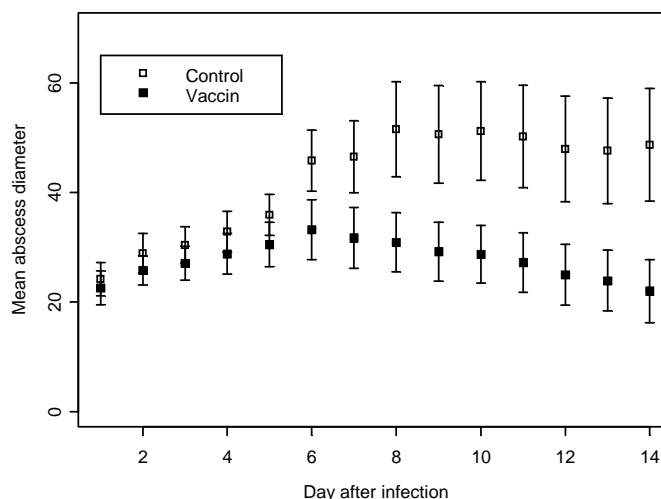
The maximum observed abscess diameter for each animal, and secondly the area under the abscess diameter curve (AUC=sum of diameters over the whole study period) were compared statistically between the two groups. For animals euthanized before the end of the study period, the LOCF (last observation carried forward) procedure was used, assigning the value of the last observation for the remaining timepoints. First a one-sided Wilcoxon rank sum test was performed to test the effect of the autovaccine. Next, a repeated measures analysis was performed based on the mixed model with animal as random effect and time, autovaccine and their interaction as fixed categorical effects.

## RESULTS AND DISCUSSION

The rabbits 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.

The animals from both groups developed an abscess on the right flank within 24 h post-infection, with a diameter varying from 8 to 40 mm. Eight days post-inoculation, one rabbit of group A and three rabbits of group B had nodules of a minimum diameter of 55 mm for three subsequent days and all of them were euthanized. All animals of group A developed abscesses on the right flank of at least 20 mm within the first week of the study, with diameters ranging from 20–65 mm. From then onwards, all abscesses started to regress. At the end of the experiment, lesion diameters varied between 4–38 mm, with six of them less than 20 mm. All animals of group B developed abscesses of at least 18 mm. Eight of them reached their maximum diameter at day 5 to day 8 after inoculation with measures ranging between 18 and 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 one day to enlarge again to 28 mm at day 4. After a second regression to 14 mm at day 12, 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. From 9 out of 10 rabbits in group A and 10 out of 10 in group B the highly virulent strain was recovered after bacterial culturing. In six animals from group A and seven animals of group B, a small abscess appeared on the lower or/and upper lips from day 7 or 8 post-infection onwards.

The mean abscess size was the largest at 6 days post-infection for group A (33.2 mm) and at 8 days for group B (55.3 mm). At the end of the experiment mean sizes were 17.2 mm for group A compared to 32.1 mm for group B. It must be underlined though that one rabbit from group A and three rabbits from group B were euthanized 8 days post-infection. For the maximum diameter, the statistical analysis showed that there was in fact a significant effect of the autovaccine ( $P=0.0477$ ). For the AUC, there was a tendency for the autovaccine to be lower ( $P=0.060$ ). With repeated measures analysis, there was also only a tendency for the main effect of the autovaccine to be lower ( $P=0.077$ ).



**Figure 1:** Daily evolution of the mean abscess diameter (mm) in vaccinated and untreated (control) rabbits

In spite of the positive effect of the administration of the bacterin on the maximum abscess size, the use of an autovaccine was not able to prevent abscess formation and the spread of the bacteria towards other body sites, such as the lips, in inoculated rabbits. Of course it must be underlined that this is an experimental model where high inoculation doses were used. Such 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. In a rabbitry it is unlikely that animals

would get infected with such a high amount of bacteria, however preliminary experiments (data not shown) have indicated that these doses are necessary to reliably reproduce skin abscesses in rabbits. The effect of such an autovaccine should therefore be studied in a field trial with longer observation periods. The use of an appropriate adjuvant or an adaptation of the vaccination schedule might also enhance the effects.

## CONCLUSIONS

It can be concluded that a positive effect might be attributed to the administration of the bacterin. However, with the vaccination schedule applied in this study, the use of an autovaccine was not able to prevent abscess formation in infected rabbits.

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