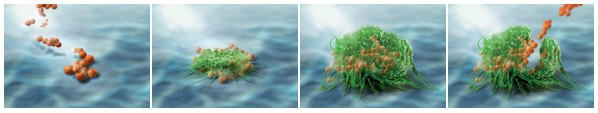
STARTVAC®

Inactivated mastitis vaccine against *E. coli*, *S. aureus*, coliforms and coagulase-negative staphylococci.

Biofilm prevention from the start



HIPRA Symposium

Results of Mastitis Vaccination

WBC'12, Lisbon

HIPRA

The **Reference**
in **Prevention**
for **Animal Health**

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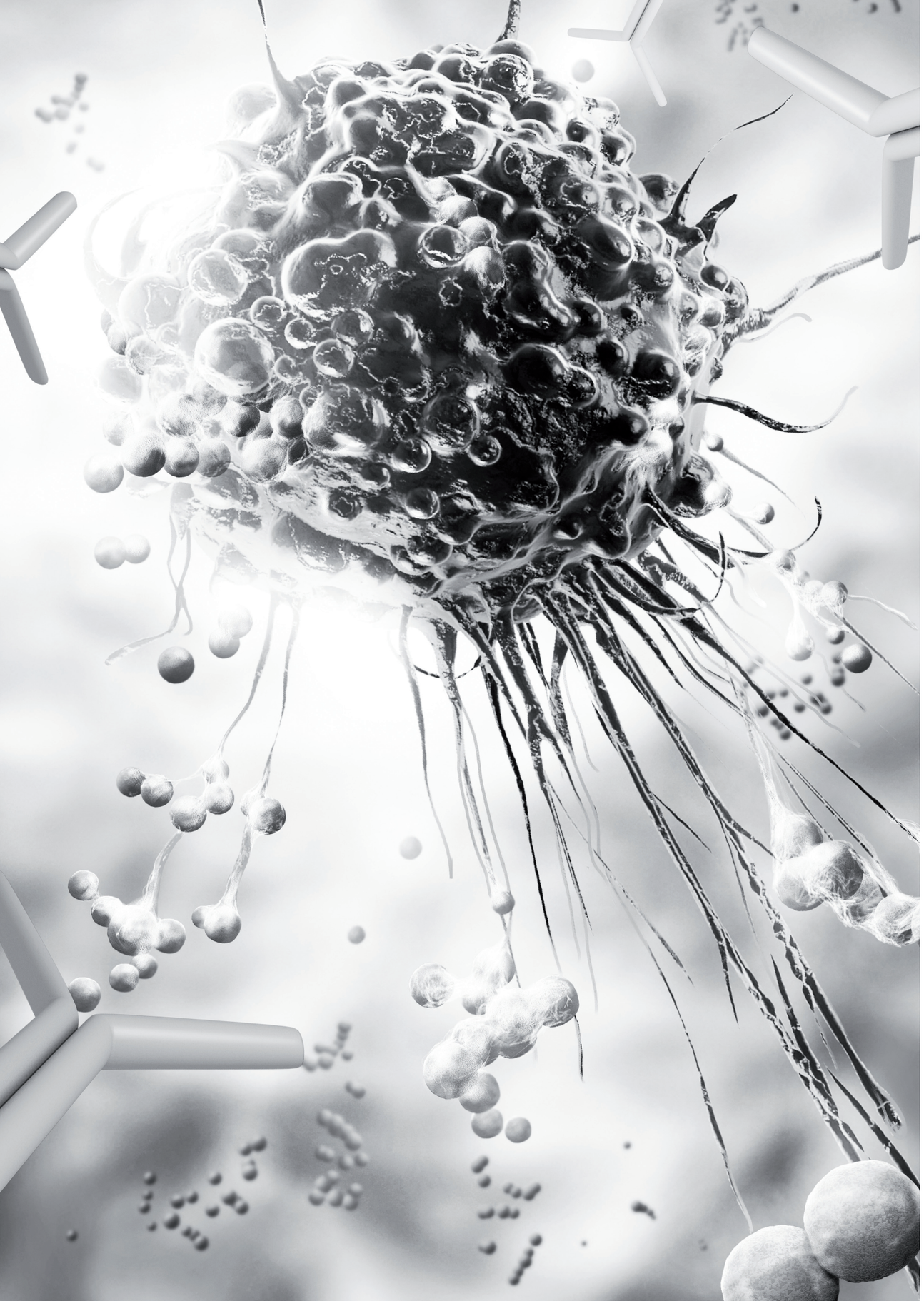

Introduction

Since its beginnings HIPRA has always been true to the philosophy and vision that defines the company: TO BECOME THE REFERENCE IN PREVENTION FOR ANIMAL HEALTH. To do so, we have invested heavily in researching and developing products that improve the health, performance and well-being of production animals. HIPRA has a wide range of vaccines for preventing and controlling diseases affecting livestock and continues working to find innovative solutions to promote continuous improvement in animal health worldwide.

Mastitis is the most important disease in the dairy cattle sector and causes significant economic losses in all farms as well as being a problem for animal welfare and which can also result in an overuse of antimicrobials. We are aware of the importance of prevention measures in controlling this disease and the need for finding new tools to improve the results obtained thus far.

Therefore, in line with our commitment to animal health and as the result of years of development, we are proud to present today the efficacy results for STARTVAC® .

STARTVAC® is the first and only vaccine registered by the European Medicines Agency (EMA) that prevents new infections by *Staphylococcus aureus*, *Escherichia coli,* coagulase-negative staphylococci and coliforms while reducing the severity of mastitis, decreasing consumption of antibiotics and lowering individual somatic cell counts. STARTVAC® prevents biofilm formation because it contains the necessary technology to induce antibodies that slow the development of the layer of biofilm-producing strains of *Staphylococcus aureus.*

Thus, we thought the best way to show you our vaccine is to present its qualities and experiences via the studies that will be presented by Sofie Piepers from the University of Ghent and Ynte Schukken from Cornell University, with the aim of making available to veterinarians a reliable, useful and effective tool that can become an integral part in the control ofthis disease.

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Bacterial biofilm

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General features

Biofilms are a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton et al., 1999). This can constitute a protected niche that allows bacteria to grow and survive in a hostile environment, particularly in environments characterized by a continuous flow. When biofilms are formed in low shear environments, they are generally more sensitive to mechanical breakage. In addition to protection against physical and chemical environmental agents, the biofilm promotes extracellular catabolism and the concentration of nutrients on cell surface.

In most natural environments, microorganisms try to adhere to available surfaces. Hence, the free-swimming (planktonic) phase can be viewed as a bacterial dispersal from one surface to colonize another. Thus, the initial phase of biofilm formation involves two stages: the first one consists in attachment of cells to a surface, facilitated by cell wall associated adhesins, which are products of various genes (Mack, 1999). Attachment to native polymeric surfaces is increased in the presence of matrix proteins including fibronectin, and fibrinogen. Following initial attachment of cells to a surface, the primary cell aggregates produce exopolysaccharides to facilitate clumping. The second stage is characterized by cell multiplication and formation of a mature structure consisting of many layers of cells, connected each other by extracellular polysaccharides (Yarwood and Schlievert, 2003). Finally, in the process of maturation, many staphylococci generate a glycocalyx, a slime layer that further protects the biofilm bacteria. The chemical nature of these slime layers is still not entirely elucidated, but evidence suggests that it consists predominantly of hydrated polysaccharides.

The growth potential of any bacterial biofilm is limited by the availability of nutrients to the cells within the biofilm and distinct flow-through channels across the biofilm aim to maintain perfusion (Stoodley et al., 2002). Other factors that are known to control biofilm maturation include internal pH, oxygen perfusion, carbon source and osmolarity (Dunne, 2002). Biofilm lives a a dynamic equilibrium and when it reaches a critical mass the outermost cell layer begins to shed planktonic organisms. These bacteria are free to escape the biofilm and to colonize other surfaces (Dunne, 2002). The formation of biofilms is often involved in the pathogenesis of many human infections caused by various microrganisms such as staphylococci, streptococci, *Ps. areuginosa, Haem. influenzae*, in many urinary infections caused by *E. coli*, as well as in infections in case of use of prostheses and implants (Hall-Stoodley et al., 2004).

Action mechanisms

Biofilm production allows bacteria to resist to antibiotic therapy, ensures infection persistence and the resistance to host immunity.

Resistance to antimicrobial agents (e.g. antibiotics) of bacteria within biofilm seems to be related to several factors: a) increased difficulty of the antibiotic to penetrate through the extracellular matrix, b) a decrease in rate of cell division (β-lactam antibiotics are effective against Gram-positive bacteria in active multiplication), c) the presence of resistant phenotypes in a bacterial population genetically heterogeneous, d) greater resistance to phagocytosis (Costerton et al., 1999). Despite some studies have reported an unimpaired antimicrobial penetration (Anderl et al., 2003), to induce the production of beta-lactamases by bacteria established in the heart of a biofilm is necessary the exposure to a higher concentration of antibiotic than in bacteria in the peripheries of biofilm (Bagge et al., 2004). Biofilm penetration of positively charged aminogylcosides is retarded by binding to negatively charged matrices, such as alginate in *Pseudomonas aeruginosa* biofilms (Walters et al., 2003). Finally, biofilm from coagulase-negative staphylococci reduced the effect of glycopeptide antibiotics, even in planktonic bacterial cultures (König et al., 2001; Souli & Giamarellou, 1998).

Resistance to host immunity contribute to maintain persistent infections. Normally planktonic bacteria are able to stimulate the production of antibodies but these are not effective against bacteria into biofilm deeper layers and may cause immune complex damage to surrounding tissues (Cochrane et al., 1998).Even in non-immunosuppressed individuals, infections caused by biofilm-producing pathogens are rarely resolved by the host defense mechanisms (Khoury et al., 1992).

All these mechanisms allow several human and animal infections to become chronic. The specific mode of growth of biofilm through release of planktonic cells is particularly related to the capability to colonize new sites and perpetuate infections.

Staph. aureus biofilm

Staph. aureus represents a major agent of contagious bovine mastitis and its ability to form biofilm suggests that it is a possible important virulence factor in the establishment of staphylococcal infection (Costerton et al. 1999).

The main constituent of the extracellular matrix, responsible for intercellular *Staph. aureus* interactions, is the exopolysaccharides poly-N-acetyl-β-1, 6 glucosamine (PNAG) synthesized by enzymes encoded from icaADBC operon. Some studies have found icaADBC operon, coding for the enzymes responsible for the biosynthesis of PNAG exopolysaccharides, in 94.36% (Cucarella et al., 2004) or in 100% (Vasudevan et al., 2003) strains of *Staph. aureus* isolated from bovine mastitis.

Besides this genetic trait, other studies have also shown a remarkable ability to produce biofilm in vitro by *Staph. aureus* isolated from cases of bovine mastitis (Vadusevan et al., 2003, Olivera et al., 2007).

The in vivo presence of the exopolysaccharides complex was also demonstrated indirectly by observing the production of specific antibodies against PNAG (Pérez et al., 2009) and SAAC (Slime Associated Antigenic Complex; Prenafeta et al., 2010) respectively in ewes and cows with experimentally induced *Staph. aureus* intramammary infections.

Vaccination against *Staph. aureus* intramammary infections

The attention paid to prevent antimicrobial resistance, particularly in meticillin-resistant *Staph. aureus* (MRSA), and a general trend, in the future, to reduce the use of antibiotics in livestock (FDA, 2010), explain the effort to develop new effective vaccines against bacterial infections.

Especially in the regards of *Staph. aureus* intramammary infections, several studies were performed to find an effective vaccine in order to decrease the spread of infection among and within herds. The targets in vaccination against mastitis are to obtain reduced inflammation at the site of injection, high efficiency against disease, a cost-efficient bacterial inoculum and an immunological parameter that could help to predict the success of vaccination (Pérez et al., 2009).

First study about vaccination against whole bacterial cells surrounded by their own biofilm matrix containing PNAG conferred protection against *Staph. aureus* infection and mastitis in a challenge study in sheep. The protection level was related to the features of the immunizing strain (degree of biofilm formation and PNAG production) and consequently to the rate of antibodies to *Staph. aureus* PNAG. Whereas of it was independent of the adjuvant and capsular polysaccharide type of the challenge strain (Pérez et al., 2009).

Further study by Prenafeta et al. in cattle (2010) has point out the active role of specific antibodies against SAAC. The immunogenicity of SAAC was demonstrated when this component was administered associated with the *Staph. aureus* bacterin in dairy heifers. Cows immunized with a greater amount of SAAC associated with the *Staph. aureus* bacterin triggered the highest SAAC-specific antibody levels in serum after vaccination. In conclusion, this study reports the immunogenicity of SAAC in dairy cows when this component is embedded in a *Staph. aureus* bacterin of a strong biofilm-producing strain and candidate it as an effective target for vaccination (Prenafeta et al.2010).

One of the benefit of using PNAG or SAAC, as antigenic component of the vaccine, is that no different serotypes have been highlighted of *Staph. aureus* in relation to the production of the two fractions mentioned above. Therefore, the antibodies induced by vaccination with these antigens give cross-protection against several strains of *Staph. aureus*.

HIPRA Symposium
Results of Mastitis Vaccination
Tuesday 5th June, WBC'12, Lisbon

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Immunological response to an experimental intramammary inoculation with a killed *Staphylococcus aureus* strain in vaccinated and non-vaccinated lactating dairy cows

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Abstract

The objective of this study was to unravel the innate immunological response after administration of a novel vaccine (Startvac® , HIPRA, S.A., Amer, Spain), containing the inactivated *Escherichia coli* J5 strain and the *Staphylococcus aureus* SP 140 strain expressing Slime Associated Antigenic Complex (SAAC). In a challenge trial, the effect of vaccination on milk neutrophil viability and concentration as well as on the antigen-specific antibodies anti-SAAC and anti-J5 was determined and several clinical parameters were observed. Eight animals were included of which four were immunized at 45 days before the expected calving date followed by a second vaccination 35 days later. The other four cows serve as non-vaccinated controls. Fifteen days after calving, two contralateral quarters of each cow were inoculated with an inactive *S. aureus* isolate. Phosphate buffered saline was administered to the two control quarters. Blood samples are collected at 45 and 10 days before calving as well as at 15 days after calving just before the infection is induced. Quarter milk samples are collected at 2 hours before, and at 4, 12, 24 and 48 hours after challenge. During the entire trial bacteriological culture and somatic cell count of the milk of all four quarters was frequently evaluated, this to exclude interference with naturally occurring intramammary infections. In conclusion, vaccinated cows seem to develop a less severe inflammatory reaction after inoculation compared to non-vaccinated animals. Vaccination also increased the level of the antigen-specific antibodies anti-SAAC and anti-J5 in blood which might eventually result in a shorter duration of the infection. However, further research is definitely needed before final conclusions on the impact of prepartum vaccination on the cows' innate immune response and their udder health status shortly after calving can be drawn.

Keywords: mastitis, vaccine, immunity

Introduction

Mastitis accounts for the largest proportion of antibiotic drug use in the dairy industry (Heringstad *et al.*, 2000). Ongoing political debates and public concerns about the emergence of antimicrobial resistance and drug residues in milk stress the need for alternatives to antibiotic therapy. In particular, the prophylactic use of antimicrobials is coming under scrutiny. One such use of antibiotics is dry cow therapy. As a consequence, there is an increasing interest in the possibilities to boost the host immune responses.

Both heifers and multiparous cows suffer from immune suppression around parturition, characterized by a higher proportion of less viable blood and milk polymorphonuclear neutrophils (PMN) (Van Oostveldt *et al.*, 2001; Mehrzad *et al.*, 2002). This phenomenon most probably explains the high incidence and increased severity of clinical mastitis in early lactation (Barkema *et al.*, 1998) as PMN play a key role in the elimination of bacteria in the early stages of intramammary infection (IMI) (Paape *et al.*, 2002).

Enhancement of the immunological response by vaccination is an attractive alternative approach for mastitis prevention and control. Prepartum vaccination did reduce the severity and duration of clinical disease post-challenge in one study (Middleton *et al.*, 2006), and had a positive effect on milk production in another study (Pellegrino *et al.*, 2008). However, little is known about the effect of vaccination on the functionality of PMN.

The aim of this study was to evaluate the effect of administration of the Startvac® vaccine (HIPRA, S.A., Amer, Spain) on milk PMN concentration and viability. Secondly, the production of the antigen-specific antibodies anti-SAAC (against *S. aureus*) and anti-J5 (against *E. coli*) in blood was determined over dry period.

Materials and Methods

Eight clinically healthy cows and heifers were selected at the research dairy farm of the Faculty of Veterinary Medicine, Ghent University, Belgium (Agri-Vet). Three animals were vaccinated intramuscularly at 45 days and 10 days before the expected calving date with the Startvac® vaccine (HIPRA, S.A., Amer, Spain) containing the inactivated *Escherichia coli* J5 strain and the *Staphylococcus aureus* SP 140 strain expressing Slime Associated Antigenic Complex (SAAC) (Prenafeta et al., 2010). At 15 days in milk (DIM), two contra-lateral quarters of each of the six cows were inoculated with the formaldehyde killed *Staphylococcus aureus* C 195 strain (HIPRA, S.A., Amer, Spain) 2 hours after morning milking. The two other quarters were inoculated with phosphate buffered saline (PBS) and served as control quarters. Duplicate quarter milk samples (5 ml) were aseptically collected for bacteriological culturing and determination of the somatic cell count (SCC) at different time points before and after inoculation (Table 1). Bacteriological culturing was performed at several time points to exclude interference with naturally occurring IMIs. Additionally, quarter milk samples (200 ml) were collected for the quantification of PMN viability at different time points between 15 and 17 DIM (Table 1).
Bacteriological culture was done as previously described (Piepers *et al.*, 2007) and performed at the lab of the Mastitis and Milk Quality Research Unit (Merelbeke, Belgium). Quarter milk SCC (qSCC) was quantified by electronic counting (Direct Cell Counter, De Laval, Gent, Belgium).

The milk used to isolate PMN was divided into several 50 ml Falcon-tubes and diluted 1:1 with PBS. All tubes were centrifuged (600×g) during 15 minutes, the cream layer and supernatant were removed, and each pellet was suspended into 10 ml PBS. Two pellets were merged together and again centrifuged (200×g) during 10 minutes, this was repeated two more times. Subsequently, milk PMN were differentiated from other milk cells by a two-step fluorescent immunolabeling using a primary anti bovine monoclonal granulocyte antibody (CH138A) (VMRD Inc., Pullman, WA, USA) and an Alexa 647 labeled goat anti mouse IgM secondary antibody (Molecular Probes, Invitrogen, Nederland) as previously described (Piepers *et al.*, 2009). To identify apoptotic and necrotic PMN, a double fluorescein isothiocyanate (FITC)-annexin-V (Roche, Indianapolis, IN, USA) and propidium iodide (PI) (Sigma-Aldrich, Bornem, Belgium) staining was used. PMN that were positive for FITC and negative for PI were considered as (early) apoptotic whereas PMN that were positive for both FITC and PI were considered necrotic. Polymorphonuclear neutrophilic leukocytes that were negative for both stains were considered viable (Piepers *et al.*, 2009; Van Oostveldt *et al.*, 2001).

Table 1: Sample overview

	DAYS BEFORE CALVING		DAYS INTO MILK							
Tasks	45d	10d	2-6d	10-14d	15-2d	15d	15+4d	15+12d	16d	17d
Vaccination[1]	×	×								
Challenge						×				
Collection of milk samples:										
- Somatic cell count			×	×	×		×	×	×	×
- Bacterial culture			×	×	×		×	×	×	×
- PMN[2]					×		×	×	×	×

[1] Three of the six cows were vaccinated.
[2] Polymorphonuclear neutrophils.

The concentration of the antigen-specific antibodies anti-SAAC and anti-J5 in blood was determined as previously described (Prenafeta *et al.,* 2010).

Linear mixed regression models adjusting for clustering of repeated measurements within quarters as well as for clustering of quarters within cows were fit to evaluate the association between the cows' vaccination status before calving and the evolution of qSCC, milk PMN concentration (Log_{10}PMN), and milk PMN viability (expressed as the proportion of viable PMN), respectively, in both the inoculated and control quarters. A similar model was fit to evaluate the association between vaccination at 45 and 10 days before calving and the concentration of the antigen-specific antibodies anti-SAAC and anti-J5.

Results and Discussion

All animals remained clinical healthy during the trial period. Challenge did not affect clinical parameters such as heartbeat rate, respiration rate, manure consistence or appetite. The average body temperature 2 hours before inoculation was 38.6°C and 38.8°C for the vaccinated and non-vaccinated animals, respectively, and did not significantly differ between both groups. In both groups, body temperature slightly increased between 15 and 17 DIM.

The average daily milk yield (MY) per cow was 33.1 liter at the onset of the trial. In the non-vaccinated group average daily MY decreased from 32.3 liter/day at 15 DIM to 27.3 liter/day at 16 DIM (P = 0.06). In the vaccinated group, no significant differences in average daily MY were observed over time. In both groups of animals, the qSCC of the challenged quarters increased over time. The difference in qSCC between the control and inoculated quarters was substantially higher in the non-vaccinated animals compared with difference in vaccinated animals (P < 0.001). Interestingly, in the vaccinated group the increase of the qSCC in the infected quarters was not significantly different from the qSCC in the control quarters (P = 0.21) (Figure 2). Similar results were obtained for the milk PMN concentration (Figure 3). The preliminary results on average daily MY and qSCC correspond well with the findings of other studies (Nickerson *et al.*, 1999; Middleton *et al.*, 2006). The difference in PMN viability between inoculated and control quarters during the trial period did not depend on the vaccination status of the animal.

The blood concentration of both anti-SAAC and anti-J5 substantially increased during dry period in the vaccinated animals only (P < 0.05). Vaccinated animals had a significantly higher anti-SAAC and anti-J5 blood concentration at the time of calving than the non-vaccinated animals (P < 0.05) (Figure 4 & 5).

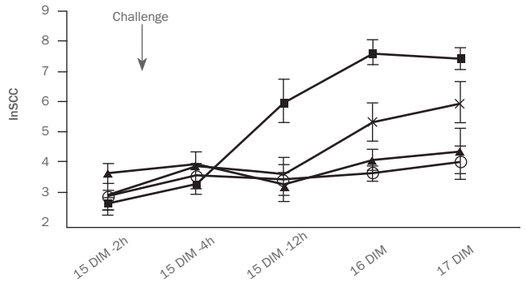

Figure 2: The evolution of the natural log-transformed quarter milk somatic cell count (qLnSCC) (± standard error) for non-vaccinated control quarters (○), vaccinated control quarters (▲),vaccinated challenged quarters (✕), and non-vaccinated challenged quarters (■).

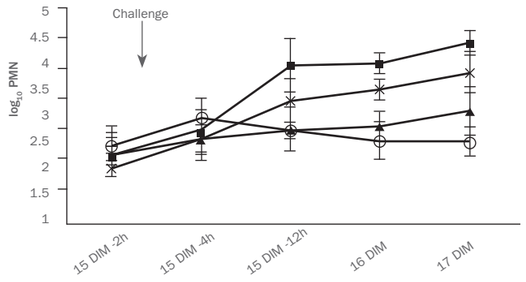

Figure 3: The evolution of the milk PMN concentration (Log_{10}PMN) (± standard error) for non-vaccinated control quarters (○), vaccinated control quarters (▲), vaccinated challenged quarters (✕), and non-vaccinated challenged quarters (■).

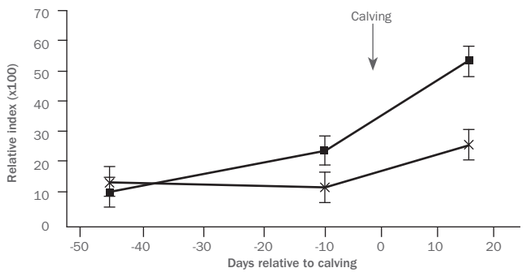

Figure 4: The evolution of the antigen-specific antibody concentration in blood (± standard error) of anti-J5 for non-vaccinated animals (✕), and non-vaccinated challenged quarters (■).

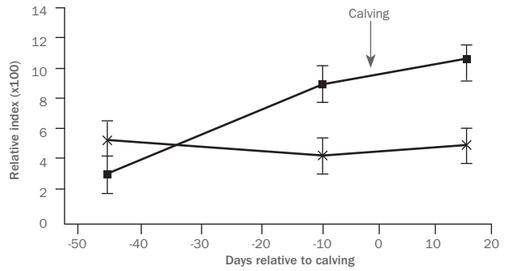

Figure 5: The evolution of the antigen-specific antibody concentration in blood (± standard error) of anti-SAAC for non-vaccinated animals (✕), and non-vaccinated challenged quarters (■).

Conclusions

Based on these preliminary results, vaccinated cows seem to undergo a less severe inflammatory reaction after inoculation compared to non-vaccinated animals. This could possibly explain why no change in daily MY was observed in the vaccinated animals, while the non-vaccinated animals suffered from a substantial drop in milk production in the days after challenge. The higher anti-SAAC and anti-J5 blood concentration might result in a more pronounced humoral specific immune response and thus eventually in a shorter duration of the infection. Further research is definitely needed before final conclusions on the impact of prepartum vaccination on the cow's innate immune response and their udder health status shortly after calving can be drawn.

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Estimation of efficacy of Startvac® vaccination in dairy herds

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Introduction

Among the bacteria that cause bovine mastitis, *Staphylococcus aureus* (*S. aureus*) plays an important role. Many infections of the mammary gland are due to this pathogen and the role of *S. aureus* in mastitis is worldwide and across many management systems. The control of *S. aureus* intramammary infections is apparently not easy and many components of mastitis control programs are necessary to fully control *S. aureus* on dairy farms (Barkema et al. 2006). Such control programs include management procedures such as optimal milking routine, post milking teat disinfection, a well functioning milking machine, segregation of known infected animals, culling of long-term affected animals, treatment of infected quarters and the use of dry cow therapy. More recently, the use of vaccines has become an additional tool in the control of *S. aureus* intramammary infections. This is especially valuable as antibiotic treatment of intramammary infections has come under scrutiny. Cell surface polysaccharides have been proposed as vaccine candidates. One of these carbohydrate antigens, poly-N-acetylglucosamine (PNAG), is a surface polymer produced by a variety of bacterial species, including *S. aureus* and S. epidermidis. PNAG is an adhesin that facilitates bacterial cell-to-cell contact in biofilms. It was recently shown that bacterins from strong biofilm-producing *S. aureus* bacteria triggered the highest production of antibodies to PNAG and conferred the highest protection against infection and mastitis following intra-mammary challenge with biofilm-producing *S. aureus* bacteria. Thus, bacterins from strong biofilm bacteria were used to develop a vaccine against *S. aureus* ruminant mastitis.

Even thought challenge trials have shown a certain degree of protection of bacterins agains the *S. aureus* challenge, the ultimate value of the vaccine will need to be shown under commercial farm conditions. Estimation of vaccine efficacy under field conditions is therefore essential. However, estimation of vaccine efficacy is complex and it is important to fully understand the potential components of vaccine efficacy that may be affected by the vaccine under consideration. In figure 1, four components of the infectious process that may be affected by a vaccine are shown in a simplified schematic. The first component is the impact of vaccinations on the rate of new infections. This represents the classic vaccine effect, whereby the vaccine reduces the susceptibility of not infected individuals such that no or fewer infections take place. The second component is the impact of vaccination on the infectiousness of an infected individual. The vaccine reduces the amount of shedding of infected but vaccinated individuals compared to non-vaccinated infectious individuals. As *S. aureus* is a mammary pathogen that may be transmitted from cow-to-cow, a reduction in the infectiousness of a vaccinated individual would be valuable. This reduction in infectiousness was also observed in the reported challenge trials (Pérez et al. 2009). The third component is the impact of vaccination on the cure of infection. Vaccinations may result in a shorter duration of infection. The duration is essentially the inverse of cure, so a higher cure will result in a shorter duration. The fourth and final component of vaccine impact is the reduction in progression of infection from subclinical to clinical mastitis. As clinical mastitis results in milk discard, treatment and animal sickness, a reduction in progression of infection would be of value to the dairy industry.

To evaluate vaccine efficacy of a *S. aureus* vaccine under field conditions, all four components of vaccine efficacy should be evaluated and preferably quantified separately. The design and analysis of vaccine evaluation studies has been the topic of many recent studies, and progress in this field of science allows the execution of field trials that are able to provide insight in most if not all component of vaccine efficacy.
In this paper, the design of a field trial for the estimation of vaccine efficacy of a new *S. aureus* vaccine will be discussed and the first preliminary results will be presented.

Figure 1. Schematic representation of the infectious processes where vaccination may play a role. Four processes are represented: susceptibility to new infections, infectiousness, cure of infection and progression to clinical disease.

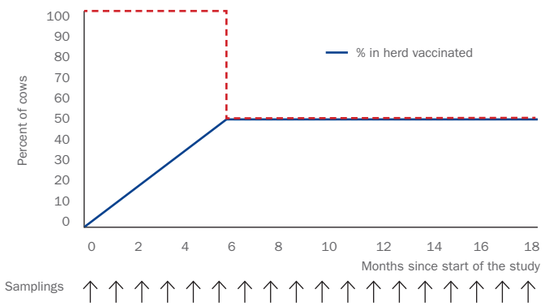
Study design

The study to estimate vaccine efficacy was a randomized negative control field trial, whereby animals in two herds were randomly assigned to either vaccination or no-treatment controls. The two dairy herds were selected based on herd size (approximately 500 lactating cows in total), known prevalence of *S. aureus*, ability to keep records, participation in dairy herd improvement monthly test day measurements and the willingness and interest of the owners to participate in the study. One of the herds was overseen by staff of Università degli Studi di Milano, the other herd was overseen by the herd's private practitioner (FT).

Vaccination of cows was done according to label, with a total of three doses of the vaccine, with the first injection at 45 days before the expected parturition date; the second injection 35 days thereafter (corresponding to 10 days before the expected parturition date); and the third injection 62 days after the second injection (equivalent to 52 days post-parturition). The full immunization program was repeated with each gestation. Both pregnant heifers and cows in lactation 1 and higher were included in the trial.

Vaccination took place according to the design shown in Figure 2. For the first 6 months, all heifers and cows in late gestation were vaccinated. After 6 months, or until approximately 50% of animals in the herd had been enrolled in the vaccination program, vaccination was done on only 50% of animals.

Figure 2. Design of a within herd randomized controlled trial to estimate the efficacy of a *S. aureus* vaccine.



By vaccinating all animals for the first 6 months, the objective of 50% vaccination was reached as fast as possible. After the initial 100% vaccination period, true randomization happened thereafter. This design allows us to evaluate vaccine efficacy starting 6 months into the study. The herds will be followed for an additional 12 months after the first period of 100% vaccination of cows in late gestation. The vaccine contains inactivated Escherichia coli (J5); inactivated *Staphylococcus aureus* (CP8) SP 140 strain expressing Slime Associated Antigenic Complex (SAAC) and adjuvant. The vaccine is administered intramuscularly. The vaccine has a label claim for reducing the incidence of sub-clinical mastitis and the incidence and the severity of the clinical signs of clinical mastitis caused by coliform, *S. aureus* and coagulase negative staphylococci. In this report we will focus on the efficacy of the vaccine against *S. aureus* only.

Sampling of all quarters of all lactating cows takes place on a monthly interval. Also, cows that have calved, dried-off, have a case of clinical mastitis or cows that are being removed from the herd are samples by herd personnel. On all samples a somatic cell count will be measured. All samples are cultured at the mastitis laboratory of Università degli Studi di Milano. All *S. aureus* and CNS isolates are frozen for further analyses. For all bacterial species, and approximate colony count will be performed. At the completion of the study, it is expected that approximately 40,000 samples will have been collected.

The ultimate outcome of the study will be an estimate of vaccine efficacy. Vaccine efficacy for susceptibility is calculated as: VE_s = 1 - Relative risk of infection in vaccinated versus controls. Similarly, the vaccine efficacy for cure is: VE_c = 1 - Relative risk of the duration of infected in vaccinated versus control. The vaccine efficacy for infectiousness and progression to clinical can be calculated.

By using a within herd randomized controlled design, vaccinated and controls cows will be comparable with regard to all housing, environment and management variables with the exception of their vaccination status. This allows for a valid comparison of vaccinated and controls. The disadvantage of such a design is the bias towards no-effect that is inherent in such a design. Because non vaccinated control cows are partly protected by their vaccinated herd mates, they will show a lower incidence of infection. At the same time, the vaccinates are exposed to more infectious material due to the fact that they are surrounded by non-vaccinated herd mates. Hence, control are less exposed and likely less infected, while vaccinates are more exposed and likely more infected compared to a situation that the whole herd was either not vaccinated or fully vaccinated. As a result the difference between vaccinated and controls is likely smaller compared to a comparison of fully

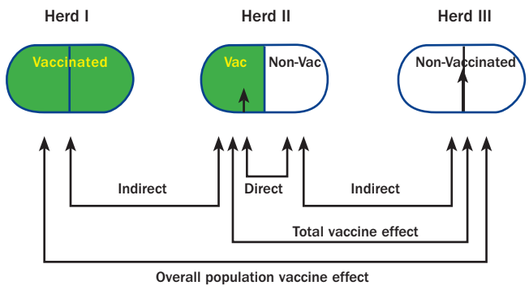
vaccinated and fully non-vaccinated herds. The difference in infection risk in a within herd randomized vaccination trial is called the direct vaccine effect. The difference in infection risk in non-vaccinated animals between a fully non-vaccinated herd and a randomized vaccinated and control herd is called the indirect vaccine effect. The sum of these two effects is called the total vaccine effect. A pictorial summary of these vaccine effect estimates is shown in figure 3. The comparison of a fully vaccinated and a fully non-vaccinated herd will allow the calculation of the overall population vaccine effect. The latter estimate is the most relevant vaccine effect when vaccinations are applied to populations of animals rather than to individual animals. Depending on the vaccine and the vaccine usage on a farm, the direct vaccine effect of the overall population vaccine effect will be the most valid estimate for a specific vaccine.

The precise field study as developed for the Startvac® vaccine will eventually allow the calculation of all four vaccine efficacy estimates (susceptibility, cure, infectiousness and progression). To allow for a correction of the direct vaccine effect for the bias towards no effect, a mathematical modeling approach will be used to obtain an unbiased estimate of vaccine efficacy. To be able to obtain an unbiased estimate, the risk of new infections in the vaccinated and non-vaccinated control population will be modeled as:

$$\text{New infections}_v = \beta_v \,.\, \#\text{negative}_v \,.\, \#\text{positive}_{v+c}$$

$$\text{New infections}_c = \beta_c \,.\, \#\text{negative}_c \,.\, \#\text{positive}_{c+v}$$

Figure 3. Study designs for vaccine efficacy estimation and the relevant vaccine effects for each study design.



The number of new infections is modeled as a function of a transmission parameter, multiplied by the number of culture negative quarters and the number of positive *S. aureus* shedding quarters. In these equations, v is for vaccinates and c is for non-vaccinated controls. The unbiased vaccine efficacy (VE) for susceptibility can then be calculated as:

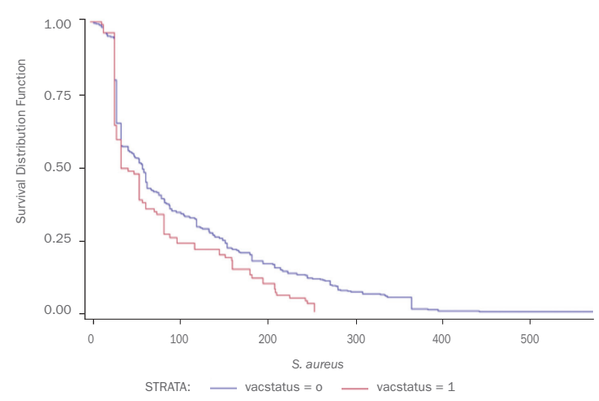
$$VE = 1 - \frac{\theta_v}{\theta_c}$$

Preliminary results

The randomized controlled field trial is approximately halfway it full length. Cows have been vaccinated for about one year and in both herds the vaccination schedule has now changed to a 50%/50% allocation of vaccinated and controls. In both herds, data is of high quality with very few missing values. Prevalence of *S. aureus* in the herd is approximately 10%, while the prevalence of coagulase negative staphylococci is approximately 5%. These relative high prevalences indicate that sufficient challenge is present in both herds.

The initial results during the first months of the valid comparison of vaccinates and controls after the start of the randomized 50%/50% vaccination schedule shows a lower incidence of new *S. aureus* infections in vaccinated animals versus control animals. These initial data show a vaccine efficacy for susceptibility of approximately .50 or 50%. No difference between vaccinated and controls is observed in average colony forming units in S. aureus infected cows. However, the average duration of infection of a S. aureus infection is shorter in the vaccinated animals compared to the non-vaccinated control animals. The difference in duration of infectious period is shown in Figure 4. A first estimate of vaccine efficacy of cure was calculated as .73 or slightly over 70%. These initial estimates of vaccine efficacy for *S. aureus* are based on relative small numbers and need to further confirmed during the remaining months of the study.

Figure 4. Time to cure or end of observation period for S. aureus infections in either vaccinated cows (red line) or non-vaccinated control cows (blue line).



Discussion and conclusions

Estimation of vaccine efficacy of contagious mastitis organisms under field conditions is an interesting challenge. The design of a randomized controlled trial is even more complicated if vaccination is limited to late gestation so that the number of vaccinated individuals increases only slowly over time. Vaccine efficacy has at least four components and intensive longitudinal studies are necessary to be able to estimate the four different components of vaccine efficacy. Ultimately all these four components will contribute to the success of a vaccine, whether measured in infection dynamics in a population or in the economic benefit of vaccination.

An intensive and large randomized field trial to evaluate the efficacy of Startvac® vaccination is described in detail. The study is currently underway and only initial estimates of vaccine efficacy can be provided. The first results indicate an acceptable vaccine efficacy for susceptibility and for cure of infection. However, several months of additional data are essential to further confirm and stabilize the initial estimates of vaccine efficacy. When the final efficacy estimates are available, further economic modeling will be possible to define the cost-benefit ratio of the Startvac® vaccination program.

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STARTVAC®

The **Reference** in **Prevention** for **Animal Health**

STARTVAC® Inactivated vaccine, Bovine mastitis, in injectable emulsion. COMPOSITION PER DOSE (2 ML): Inactivated *Escherichia coli* (J5) 50 RED_{60}*; Inactivated *Staphylococcus aureus* (CP8) SP 140 strain expressing SAAC** 50 RED_{80} ***. Adjuvant. * RED_{60}: Rabbit effective dose in 60% of the animals (serology). **SAAC: Slime Associated Antigenic Complex. *** RED_{80}: Rabbit effective dose in 80% of the animals (serology). **PROPERTIES**: Mastitis is one of the mail problems in dairy cows, not only from an economic point of view due to losses in the quantity and quality of the milk, but also from a sanitary point of view, because the milk produced has low bacteriological quality and a high level of antibiotics, as a consequence of antimastitis treatments. The vaccine STARTVAC, which combines specific antigens and a special adjuvant, prevents and minimizes the effects of mastitis caused by *Staphylococcus aureus* (the main responsible for chronic mastitis) and *Escherichia coli* (causative agent of acute clinical mastitis). **INDICATIONS: Cows and Heifers:** To prevent Mastitis. For herd imminisation of healthy cows and heifers, in dairy cattle herds with recurring mastitis problems, to reduce the incidence of sub-clinical mastitis and the incidence and the severity of the clinical signs of clinical mastitis caused by *Staphylococcus aureus*, coliforms and coagulase-negative staphylococci. The full inmunisation scheme induces immunity from aproximately day 13 after the first injection until approximately day 78 after the third injection (equivalent to 130 days post-partirition). **SIDE EFFECTS:** Slight to moderate transient local reactions may occur after the administration of one dose of vaccine, which disappears within 1 or 2 weeks at most. **ADMINISTRATION ROUTE:** Intramuscular, into the neck muscle. The injections should be preferably administered on the alternate sides of the neck. It is advisable to administer the vaccine at a temperature between +15 and +25 ºC. Shake before use. **DOSAGE**: **Cows and Heifers**: 2 ml/animal. Generally, the following vaccination programme is recommended: *First injection:* at 45 days before the expected parturition date. *Second injection*: 35 days thereafter (corresponding to 10 days the expected parturition date). *Third injection*: 62 days after the second injection (equivalent mastitis control program that addresses all important udder health factors (e.g. milking technique, dry-off and breeding management, hygiene, nutrition, bedding, cow confort, air and water quality, health monitoring) and other management practices. Can be used during pregnancy and lactation. **WITHDRAWAL PERIOD: 0 days. SPECIAL PRECAUTIONS:** Store at +2 to +8 ºC, avoiding freezing. Protect from light. **PACKAGING**: Pack of 20 vials of 1 ds. 5 ds vial. 25 ds bottle. Under veterinary prescription. Marketing authorisation holder: Laboratorios Hipra, S.A. la Selva, 135, 17170-AMER (Girona) SPAIN. Marketing authorisation numbers: 1 dose: (EU/2/08/092/003); 5 doses: (EU/2/08/092/006). Use medicines responsibly.

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