STARTVAC® VACCINATION AGAINST MASTITIS: ESTIMATION OF EFFICACY IN DAIRY HERDS AND IMMUNOLOGICAL RESPONSE

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Introduction

Mastitis affects a high proportion of cows throughout the world and is without doubt still one of the most costly diseases for the dairy industry (Bradley, 2002). The financial losses associated with mastitis are mainly incurred by milk production losses, treatment costs, and culling (Huijps *et al*., 2008). Additionally, farmers supplying milk with high bulk milk somatic cell count may be losing out on bonus payments as well as incurring penalties. Mastitis also accounts for the largest proportion of antibiotic drug use in the dairy industry, strongly harming the image of milk as a high quality product. Indeed, herds with higher bulk milk somatic cell count have a higher risk of antibiotic residue violation because of their increased antibiotic usage (Ruegg and Tabone, 2000). Clinical mastitis has, in addition, its implications for animal welfare (Bradley, 2002). Treating infected cows also increases labor usage (e.g. time and efforts) and causes stress of which the consequences should not be underestimated as they are both perceived as the two most annoying aspects of mastitis by farmers (Jansen *et al*., 2009).

Among the bacteria that cause bovine mastitis, *Escherichia coli (E. coli)* and *Staphylococcus aureus* (*S. aureus*) play an important role. *Escherichia coli* is often involved in hyperacute clinical mastitis cases characterized by abnormal appearance of milk, hard mammary quarters, depressed appetite, reduced milk production, and in worst case scenario dehydration, recumbency, and death. Curative therapy with antibiotics remains only moderately effective and depends on the severity and stage at which the disease is treated. The most successful strategies for preventing and controlling coliform mastitis rely on improving the hygienic management. The severity of clinical symptoms can be reduced by prophylactic immunization with the *E.* coli J5 vaccine (Wilson and Gonzalez, 2003). The role of *S. aureus* in mastitis is worldwide and across many management systems. Intramammary infections with *S. aureus* are apparently not easy to control 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, and 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 as well. This is especially valuable as antibiotic treatment of intramammary infections has come under scrutiny.

In essence, vaccination is a form of active immunization entailing the introduction of a foreign molecule, e.g. bacteria or parts of the bacteria into the cow causing the cow itself to generate immunity via the production of antibodies specifically oriented against the target. Using this

binding mechanism, an antibody can “tag” the bacteria for attack by other parts of the cow’s immune system such as macrophages and neutrophils, or can neutralize its target directly e.g. by blocking a part of the microbe that is essential for either its invasion or survival. Vaccines against *E. coli* primarily contain the inactive J5 *E. coli* strain, resulting in the formation of antibodies against the uniform component lipopolysaccharide (LPS) in the outer membrane of Gram-negative bacteria causing the severe symptoms associated with hyperacute *E. coli* mastitis cases. The latter explains why vaccination with J5-containing vaccins generally results in a less severe and shorter inflammatory response (Wilson and Gonzalez, 2003). Vaccines against *S. aureus* consist of either bacterins (= killed or avirulent/weakend *S. aureus* strains) or exopolysaccharides (= sugar residues secreted by bacteria in the surrounding environment). One of those exopolysaccharides is poly-N-acetylglucosamine (PNAG), a surface polymer produced by a variety of bacterial species, including *S. aureus* and *Staphylococcus epidermidis*. PNAG is an adhesion 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 intramammary challenge with biofilm-producing *S. aureus* bacteria. Thus, bacterins from strong biofilm bacteria were used to develop the novel vaccine Startvac® against *S. aureus* ruminant mastitis.

The novel vaccine Startvac® contains inactivated *E. coli* (J5), inactivated *S. aureus* (CP8) SP 140 strain expressing Slime Associated Antigenic Complex (SAAC) and adjuvant. The vaccine has a label claim for reducing the incidence of subclinical mastitis and the incidence and severity of the clinical signs of clinical mastitis caused by coliform, *S. aureus*, and coagulase-negative staphylococci (CNS). Even though challenge trials have shown a certain degree of protection against *E. coli* and *S. aureus* bacteria, 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. Also, the immunological basis of its mechanism is still unknown. Hypothetically, protection by vaccination could be the result of an increased opsonization via increased antibody concentrations in blood and milk and eventually a more efficient phagocytosis and killing of bacteria. Direct enhancement of the polymorphonuclear neutrophilic leukocyte (PMNL) viability and activity could be another potential mechanism of action. Although both hypotheses are plausible, none of them has yet been truly investigated.

In this report, we will focus on the efficacy of the vaccine against *S. aureus* only and the differences in immunological response between vaccinated and non-vaccinated animals. The design of a field trial for the estimation of the efficacy of the new vaccine against *S. aureus* will be discussed and the first preliminary results will be presented. Also, the effect of administration of the novel vaccine on the immunological response to an experimental intramammary inoculation with a killed *S. aureus* strain in vaccinated and non-vaccinated lactating dairy cows is described. Preliminary results of the efficacy of the vaccine against *E. coli* in the field will be presented at the conference.

Efficacy of Startvac® vaccination against *S. aureus*

Background

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.

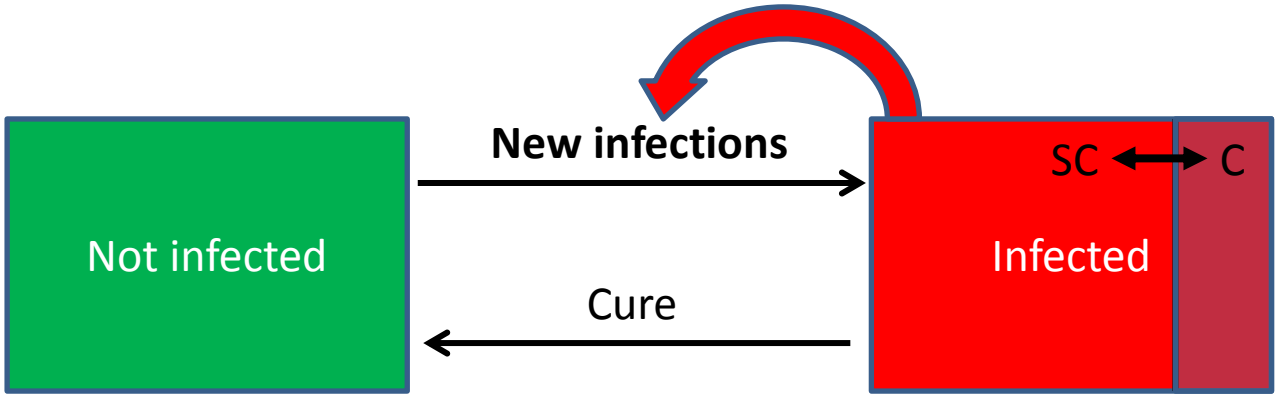

Figure 1. Schematic representation of the infectious processes where vaccination may play a role. Four processes are represented: susceptibility to new infections, infectiousness, cure of infection and progression to clinical disease.

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 (480 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.

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 another 12 months after the first period of 100% vaccination of cows in late gestation. The vaccine is administered intramuscularly.

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 sampled 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.

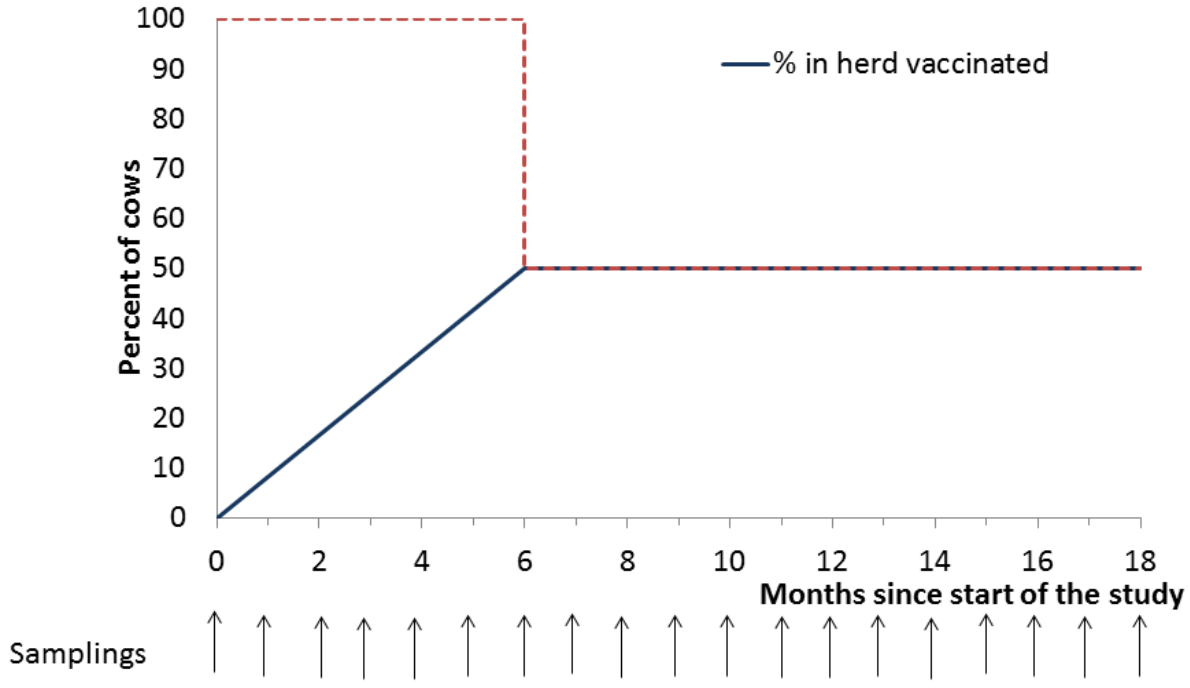

Figure 2. Design of a within herd randomized controlled trial to estimate the efficacy of a *S. aureus* vaccine.

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.

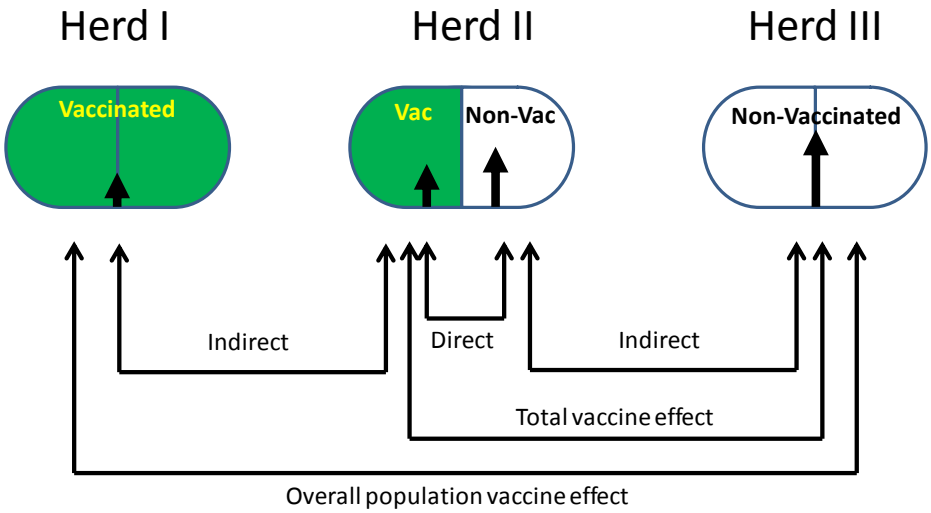

Figure 3. Study designs for vaccine efficacy estimation and the relevant vaccine effects for each study design.

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:

New infections$_v$ = β_v . #negative$_v$. #positive$_{v+c}$

New infections$_c$ = β_c . #negative$_c$. #positive$_{c+v}$

The number of new infections is modeled as a function of a transmission parameter, β, 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:

$$VE = 1 - \frac{\beta_v}{\beta_c}$$

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 CNS 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 .57 or 57%. 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.

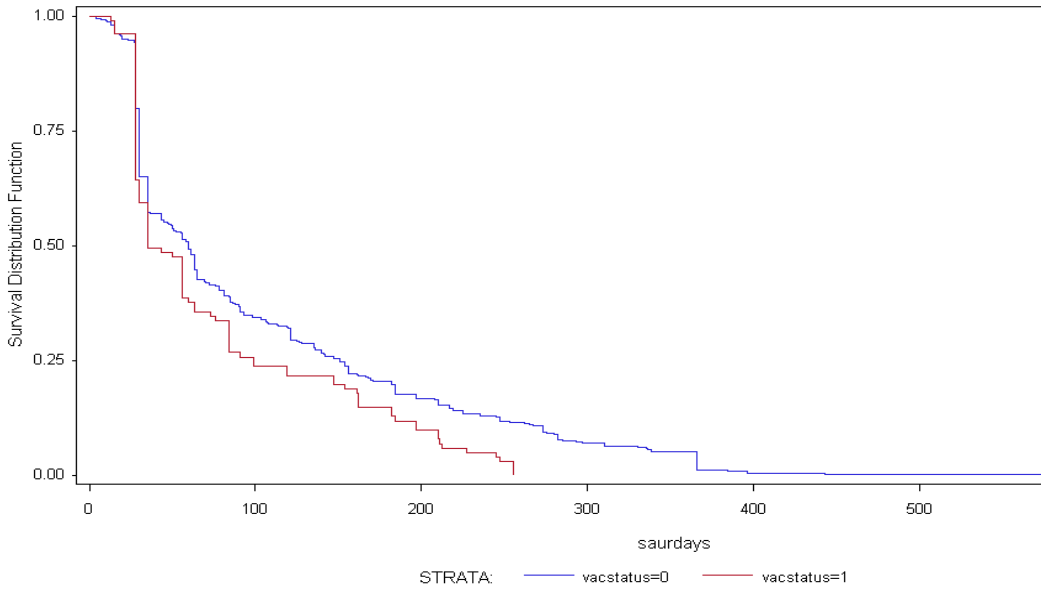

Figure 4. Time to cure or end of observation period for *S. aureus* infections in either vaccinated cows (red line) or non-vaccinated control cows (blue line).

Immunological response to a killed *S. aureus* strain

To unravel the (innate) immunological response after administration of the novel vaccine, a challenge trial was set-up. In that trial, the effect of vaccination on milk PMNL viability and concentration as well as on the antigen-specific antibodies anti-SAAC and anti-J5 was determined and several clinical parameters were observed.

Study design

Eight clinically healthy cows and heifers were selected at the research dairy farm of the Faculty of Veterinary Medicine, Ghent University, Belgium (Agri-Vet). Four 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 eight 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 intramammary infections. Additionally, quarter milk samples (200 ml) were collected for the quantification of PMNL viability at different time points between 15 and 17 DIM (Table 1).

Table 1: Sample overview

	Days before calving		Days into milk							
Tasks	45d	10d	2-6d	10-14d	15d -2h	15d	15d +4h	15d +12h	16d	17d
Vaccination[1]	×	×								
Challenge						×				
Collection of milk samples:										
- Somatic cell count			×	×	×		×	×	×	×
- Bacterial culture			×	×	×		×	×	×	×
- PMNL[2]					×		×	×	×	×

[1] Four of the eight cows were vaccinated.

[2] Polymorphonuclear neutrophilic leukocytes

Laboratory analyses

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).

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

Statistical analyses

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 PMNL concentration (Log_{10}PMNL), and milk PMNL viability (expressed as the proportion of viable PMNL), 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

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 33.2 liter at the onset of the trial. In the non-vaccinated group average daily MY decreased from 34.2 liter/day at 15 DIM to 30.5 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 ($P < 0.05$). 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 (Figure 5). Similar results were obtained for the milk PMNL concentration (Figure 6). 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 PMNL 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 7). The milk concentration of anti-SAAC from 15 up to 17 DIM was significantly higher in vaccinated animals than in non-vaccinated animals, independently from the infection status of the quarters ($P < 0.05$). Although from 15 up to 17 DIM a numerically higher milk concentration of anti-J5 was observed in vaccinated than in non-vaccinated animals, the difference was not significant.

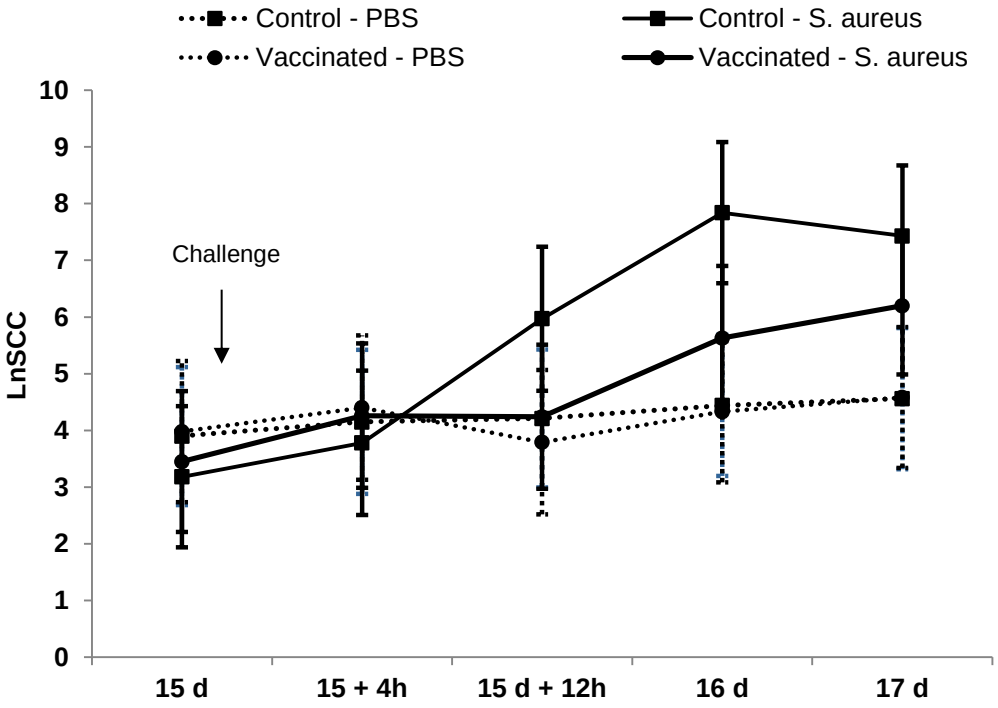

Figure 5: The evolution of the natural log-transformed quarter milk somatic cell count (qLnSCC) (± standard error) for non-vaccinated control quarters, vaccinated control quarter, vaccinated challenged quarters, and non-vaccinated challenged quarters.

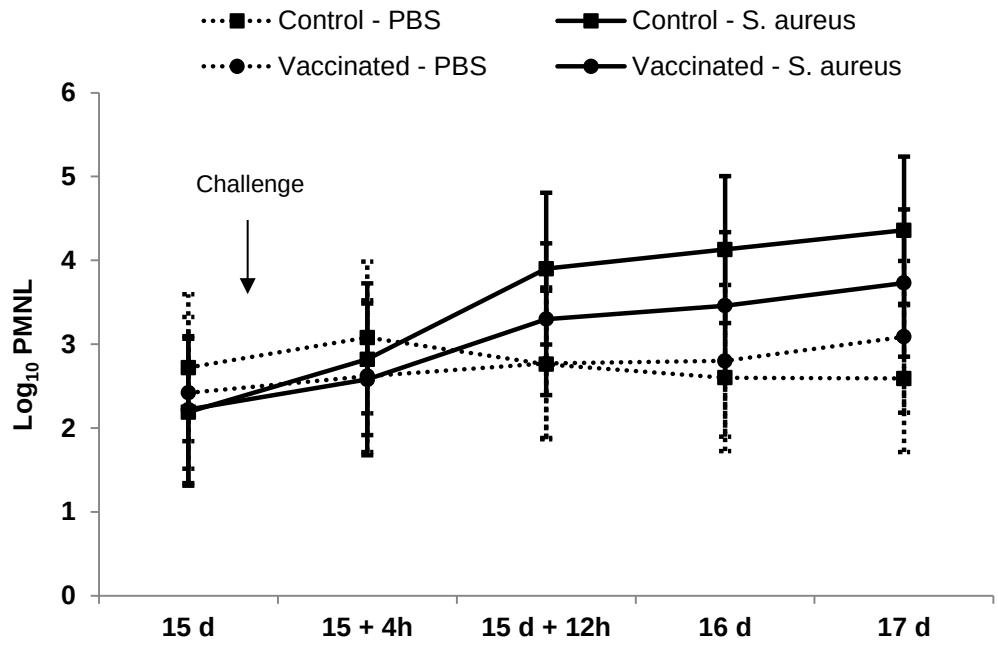

Figure 6: The evolution of the log_{10}-transformed quarter concentration of polymorphonuclear neutrophilic leukocytes (Log_{10}PMNL) (± standard error) for non-vaccinated control quarters, vaccinated control quarter, vaccinated challenged quarters, and non-vaccinated challenged quarters.

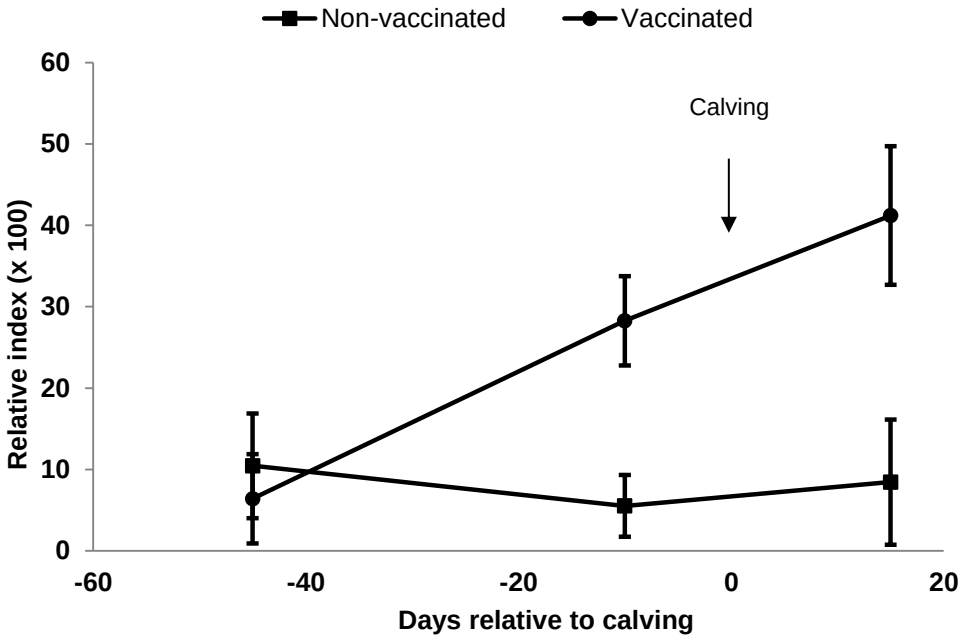

Figure 7: The evolution of the antigen-specific antibody concentration in blood (± standard error) of anti-SAAC for non-vaccinated animals and vaccinated animals.

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. The first results of a large longitudinal field trial indicate an acceptable efficacy of the Startvac® vaccine for susceptibility and cure of intramammary infections with *S. aureus*. 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. Preliminary results of the efficacy of the novel vaccine against *E. coli* in dairy herds will be presented at the conference.

Based on the preliminary results of the challenge trial, 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 first 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. Also, the higher anti-SAAC concentrations in milk might trigger the opsonization of the inoculated *S. aureus* bacteria and partly explain why vaccinated animals suffered from a less severe inflammatory reaction than the non-vaccinated animals.

Further research is definitely needed before final conclusions on the impact of vaccination in late gestation with the novel vaccine Startvac® on the cows' (innate) immune response and their susceptibility for new intramammary infections and cure of intramammary infections can be drawn.

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