

**Sofie Piepers**

Ghent University



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

S. Piepers, DVM, PhD, K. Deberdt, DVM, A. De Visscher, DVM, J. Verbeke, DVM, and S. De Vlieghe, DVM, PhD

M-team and M2QRU, Department of Reproduction, Obstetrics, and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium  
Sofie.Piepers@Ugent.be

## Abstract

The objective of this study was to unravel the innate immunological response after administration of a novel vaccine (Startvac<sup>®</sup>, 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

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<sup>®</sup> 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<sup>®</sup> 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 contralateral 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

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

<sup>1</sup> Three of the six cows were vaccinated.

<sup>2</sup> 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 ( $\text{Log}_{10}$  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).

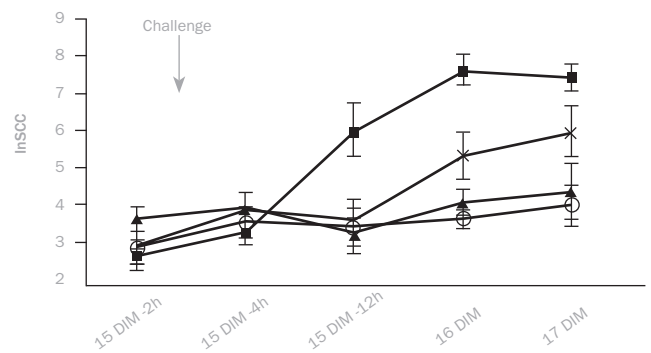


Figure 2: The evolution of the natural log-transformed quarter milk somatic cell count (qLnSCC) ( $\pm$  standard error) for non-vaccinated control quarters (○), vaccinated control quarters (▲), vaccinated challenged quarters (×), and non-vaccinated challenged quarters (■).

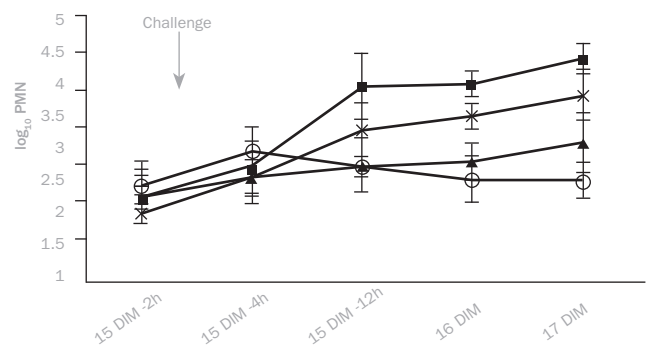


Figure 3: The evolution of the milk PMN concentration ( $\text{Log}_{10}$  PMN) ( $\pm$  standard error) for non-vaccinated control quarters (○), vaccinated control quarters (▲), vaccinated challenged quarters (×), and non-vaccinated challenged quarters (■).

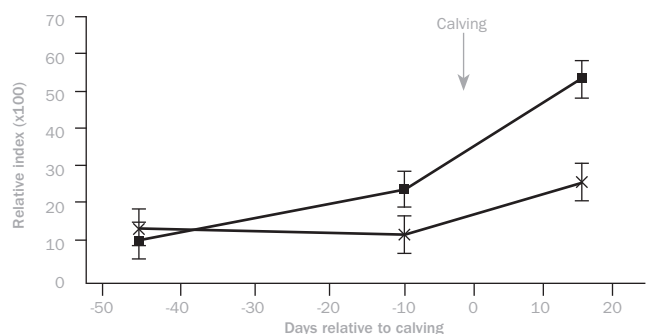


Figure 4: The evolution of the antigen-specific antibody concentration in blood ( $\pm$  standard error) of anti-J5 for non-vaccinated animals (×), and non-vaccinated challenged quarters (■).

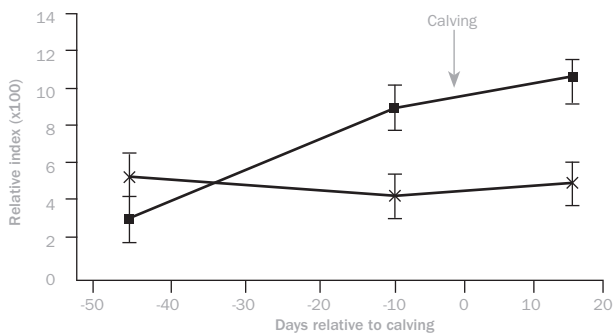


Figure 5: The evolution of the antigen-specific antibody concentration in blood ( $\pm$  standard error) of anti-SAAC for non-vaccinated animals (X), and non-vaccinated challenged quarters (■).

## 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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