Heifer and quarter characteristics associated with periparturient blood and milk neutrophil apoptosis in healthy heifers and in heifers with subclinical mastitis

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ABSTRACT

Polymorphonuclear neutrophilic leukocytes (PMNL) play an important role in the first line cell-mediated immune defense of the body in general and of the mammary gland against mastitis pathogens in particular. Reduced viability of PMNL close to parturition may explain the high incidence of infectious diseases and the high prevalence of intramammary infections (IMI) in periparturient dairy heifers. Apoptosis of blood PMNL 1 wk before the expected calving date and of blood and milk PMNL at 1 to 4 d in milk was determined using flow cytometry. Information on heifer and gland characteristics was collected before calving and in early lactation. Data were analyzed using multivariable, multilevel regression analysis. Supplementation of a commercial mineral/vitamin mix before calving was associated with less blood (14.4 ± 2.9 vs. $22.4 \pm 2.1\%$) and milk PMNL apoptosis (19.0 ± 1.1 vs. $26.4 \pm 0.9\%$) near calving, presumably related to higher blood selenium concentrations. Both blood and milk PMNL apoptosis showed seasonal variation with the highest proportion of apoptotic cells between January and March (32.0 ± 6.1 and $34.6 \pm 2.7\%$, respectively) and April and June (31.3 ± 5.7 and $37.8 \pm 2.3\%$, respectively). Heifers losing 0.25 points or more of their body condition in the periparturient period had higher proportions of apoptotic blood PMNL in early lactation compared with heifers losing less than 0.25 points (24.0 ± 2.8 vs. $16.6 \pm 1.7\%$). Milk PMNL apoptosis was less pronounced in quarters having teat orifices colonized with non-aureus staphylococci before calving (18.9 ± 1.0 vs. $29.4 \pm 1.0\%$). The variation in blood PMNL apoptosis before and after calving mainly resided at the heifer level (71.4 and 98.4% of the total variation, respectively), whereas the variation in milk PMNL apoptosis mainly resided at the heifer (45.7% of the total variation) and quarter

levels (45.5% of the total variation). These data imply that the impaired blood and milk PMNL viability in periparturient heifers can be reduced by optimization of certain heifer management practices such as supplementation of minerals/vitamins, and pasture and feeding strategies.

Key words: early lactation, heifer, milk, neutrophil apoptosis

INTRODUCTION

A high proportion of dairy heifers calve with subclinical mastitis (Fox, 2009), which is not without consequences. Indeed, IMI in early lactation have deleterious effects on future udder health, milk production, and longevity (De Vliegher et al., 2005). The impact of those IMI on the future performance of the affected heifer is almost certainly associated with the persistence of the IMI once milk production starts (Piepers et al., 2009a). Some heifers seem to eliminate IMI present at calving shortly thereafter, whereas others obviously do not. This difference may be because of variation in virulence of the pathogen and its ability to cause persisting infections. Still, the immune status of the host may play an important role. This was supported by identical coagulase-negative staphylococcal strains causing both transient and persistent IMI in heifers (Taponen et al., 2007).

The number and quality of resident and incoming PMNL were of importance as first line cell-mediated immune defense of the mammary gland (Mehrzhad et al., 2004). Heifers suffer from impaired blood and milk PMNL viability near calving (although less pronounced than in multiparous cows) via increased cell apoptosis compared with mid and late lactation (Van Oostveldt et al., 2001). This decrease in cell viability is closely related to impaired cell activity (Mehrzhad et al., 2004). Differences in the number and viability of PMNL at the heifer and quarter levels could at least partially explain why IMI occur and persist in a specific heifer or even a quarter within a heifer or cow.

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Only a limited number of factors are currently associated with the immunity of heifers in early lactation. Supplementation with minerals and vitamins, in particular with selenium and vitamin E, has a protective effect on immune cell activity and udder health in multiparous cows, but has been studied only rarely in heifers (Coe et al., 1993). Epidemiological studies identifying risk factors associated with bovine PMNL quality have focused mainly on blood PMNL, not on milk PMNL (Hogan et al., 1990). This might be because, compared with blood, milk is a difficult medium to analyze because of its high content of fat, protein, and cell debris. Recently, a flow cytometric technique was developed to accurately determine milk PMNL viability in a large number of milk samples (Piepers et al., 2009b). Knowledge of the factors associated with the viability of both bovine blood and milk PMNL could be used to implement measures to enhance the natural resistance against IMI and to stimulate the clearance of bacteria in heifers around calving.

The main objective was to identify heifer and quarter characteristics associated with periparturient blood and milk PMNL apoptosis. These associations could be helpful in improving periparturient immunity of dairy heifers or could generate new hypotheses for future research. Additionally, the contribution of herd, heifer, and quarter to the total variance of blood and milk PMNL apoptosis of periparturient dairy heifers was determined.

MATERIALS AND METHODS

The study was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2006/002).

Data

Herds and Heifers. Nineteen dairy herds were randomly selected from a database of the Flemish Cattle Breeding Association (Oosterzele, Belgium) including all dairy herds ($n = 241$) within a 30-km radius of the Faculty of Veterinary Medicine (Merelbeke, Belgium) using the Excel RAND function (Excel 2007, Microsoft Corp., Redmond, WA). Herds treating heifers with antibiotics before calving were not included, although none of the selected herds needed exclusion for this reason. Eighty-two clinically healthy heifers (average of 4 heifers per herd, ranging from 1 to 7) were randomly selected. Heifers with general signs of disease or with signs of clinical mastitis in early lactation were excluded. The heifers were kept indoors between October and June and had access to pasture between June and October. During the indoor period, heifers were fed

grass and corn silage and hay. In 7 herds, a commercial vitamin-mineral mix containing vitamin E, selenium, magnesium, zinc, and copper was fed daily to some heifers ($n = 16$) for the last 6 wk before calving (range 4 to 8 wk).

Sample Collection. To determine blood PMNL viability, blood was collected at approximately 1 wk before expected calving date and between 1 and 4 DIM (Table 1) from the tail vein using EDTA vacutainer tubes (Terumo, Leuven, Belgium) and mixed with 30 mL of Alsever's solution as anticoagulant in a 60-mL syringe. Milk PMNL viability and the approximate concentration were determined once in quarter foremilk samples at 2 DIM on average (Table 1). Data on heifer and quarter characteristics were recorded approximately 1 wk before the expected calving date, at calving, or in the first days after calving (Table 1). Body condition and cleanliness of the heifers were scored visually. Body condition was scored on a 5-point scale divided into quarter-point increments (Edmondson et al., 1989). Hygiene scores ranging from 1 (clean) to 5 (dirty) were assigned for 4 body areas: tail head, thigh, udder, and hind limbs (Hughes, 2001). Heifers with an average cleanliness score ≤ 1.7 (median value) were considered "clean" and heifers with a cleanliness score > 1.7 were considered "dirty." The presence of udder edema was scored based on Dentine and McDaniel (1983). A score of 1 (no edema), 2 (slight edema), 3 (moderate edema), 4 (severe edema), or 5 (extremely severe edema) was assigned to each heifer. None of the heifers had extremely severe edema. An alternate binary variable was used for the analyses (0 = no or slight edema; 1 = moderate to severe edema). Swabbing of the teat apices was done as described previously (De Vliegher et al., 2003). The presence of teat end lesions and teat skin lesions were recorded based on a visual scoring system (Neijenhuis et al., 2000). Quarter milk samples for bacteriological culturing (5 mL) were collected twice postpartum (Table 1) after the first streams of milk were discarded and after teat ends had been disinfected with cotton swabs drenched in 70% denatured alcohol. At approximately 1 wk before the expected calving date, additional blood samples were collected from a subset of 60 heifers using vacutainer tubes containing a polyolefin gel and silica clot activator (Terumo) to determine the serum selenium (Se) concentration.

Blood and Milk PMNL Apoptosis

Quantification of PMNL apoptosis was performed as described by Piepers et al. (2009b). Briefly, isolation of blood leukocytes was performed by centrifugation ($300 \times g$), removal of plasma and buffy coat, hypotonic lysis of erythrocytes, and 2 washing steps. The initial volume

Table 1. Overview of the variables collected from heifers associated with periparturient blood and milk neutrophil apoptosis

Description	Level	Time of sampling/recording			
		Before calving ¹	At calving	1 to 4 DIM	5 to 8 DIM
Dependent variables					
Blood PMNL apoptosis	Heifer	×		×	
Milk PMNL apoptosis	Quarter			×	
Milk PMNL concentration	Quarter			×	
Independent variables					
Access to pasture (yes/no)	Heifer	×			
Age at calving (mo)	Heifer		×		
Body condition loss ²	Heifer	×			×
Blood Se concentration ³	Heifer	×			
Breed ⁴	Heifer	×			
Cleanliness of the heifer before calving ⁵	Heifer	×			
Contact with lactating cows (yes/no)	Heifer	×			
Moderate to severe udder edema (yes/no)	Heifer			×	
Season of calving ⁶	Heifer		×		
Supplementation with minerals/vitamins (yes/no)	Heifer	×			
Infection status in early lactation ⁷	Quarter			×	×
Quarter position ⁸	Quarter	×			
Teat end lesions (yes/no) ⁹	Quarter	×			
Teat skin lesions (yes/no)	Quarter	×			
Teat apex colonization with non-aureus staphylococci (yes/no)	Quarter	×			

¹Approximately 1 wk before the expected calving date.

²Body condition loss of at least 0.25 points between first and second recording (Edmondson et al., 1989).

³Determined on a subset of 60 heifers.

⁴Black and White Holstein-Friesians, Red Holstein-Friesians, and Red Holstein-Friesian crossbreds.

⁵Based on the visual cleanliness scoring system of Hughes (2001).

⁶January–March, April–June, July–September, and October–December.

⁷See text for details.

⁸Front versus rear quarters.

⁹Based on the visual scoring system of Neijenhuis et al. (2000).

of quarter foremilk was divided equally into two 50-mL Falcon tubes, diluted 50% (vol/vol) with cold PBS, and centrifuged ($300 \times g$) for 15 min. After removing the cream layer, the pellet was suspended in 10 mL of PBS, centrifuged at $200 \times g$ for 10 min, and washed twice.

Blood PMNL were discriminated from other blood cells based on their size and internal complexity in a forward-side scatter dot plot (Van Oostveldt et al., 2001). Differentiation between milk PMNL and other milk cells was conducted by a 2-step fluorescent immunolabeling using a primary anti-bovine monoclonal granulocyte antibody (CH138A) and an Alexa 647-labeled goat anti-mouse IgM secondary antibody (Piepers et al., 2009b).

Apoptotic PMNL were identified using a double fluorescein isothiocyanate (FITC)-annexin-V and propidium iodide (PI) staining (Van Oostveldt et al., 2001; Piepers et al., 2009b). Spontaneous apoptosis in blood was induced by incubating the blood cell pellet for 18 h at 37°C before PMNL viability was determined (Van Oostveldt et al., 2001). Polymorphonuclear neutrophilic leukocytes that were positive for FITC and negative for PI were considered (early) apoptotic, whereas PMNL

that were positive for both FITC and PI were considered necrotic (Van Oostveldt et al., 2001; Piepers et al., 2009b). The latter subpopulation was excluded from the analysis. All analyses were performed on a double-laser, bench-top flow cytometer (BD FACSCanto II) and processed using FACSDiva software (both from Becton Dickinson, Erembodegem, Belgium). A square-root transformation of the proportion of apoptotic blood and milk PMNL was used to normalize the data.

Approximate Milk PMNL Concentration

Milk PMNL concentration was approximated based on the absolute number of milk PMNL events determined by flow cytometry, the sample acquisition time, and the flow rate (FR, $\mu\text{L/s}$). The FR was calculated by use of calibrator microfluorospheres (BD TruCount Controls, Becton Dickinson, San Jose, CA) with a bead count provided by the manufacturer. The bead population was defined and gated using a dot plot of FL1 (530/30 nm) versus FL2 (585/42 nm) in which a rectangular gating region was drawn to enclose the beads and exclude debris. A TruCount tube was run

at the beginning and end of each batch of samples that contained 2 negative control samples, 1 per quarter for measuring milk cell background fluorescence and 1 per animal for measuring the nonspecific binding of the fluorescent-labeled secondary antibody, and 1 test sample per quarter labeled with CH138A and Alexa 647. The mean FR was used to determine the acquired sample volume during flow cytometric analysis of the test samples (V_{acq}) using $V_{\text{acq}} = (\text{FR} \div 60 \text{ s}) \times t_{\text{acq}}$, where t_{acq} is sample acquisition time in seconds. The approximate milk PMNL concentration was calculated by using the following formula: Approximate milk PMNL concentration = $\log_{10}[(n_{\text{PMNL}} \times V_i) \div (\kappa \times V_{\text{cell}} \times V_m)]$, where n_{PMNL} = absolute number of milk PMNL events, V_i = volume of isolated cell pellet, V_{cell} = volume of cell suspension containing 2×10^5 cells (Piepers et al., 2009b), V_m = total volume of milk sample, and $\kappa = V_{\text{acq}} \div$ the total volume of diluent before acquisition.

Blood Selenium

The serum Se concentration before calving was analyzed by means of inductively coupled plasma mass spectrometry (Slovak Academy of Sciences, Kosice, Slovakia; Sieniawska et al., 1999). The reference value for the serum Se concentration was set at 60 $\mu\text{g/L}$ (Radostits et al., 2000).

Bacteriological Culturing

Bacteriological culturing and isolate identification of both milk (Animal Health Service Flanders, Torhout, Belgium) and swabbing samples (Faculty of Veterinary Medicine, Merelbeke, Belgium) were done as previously described (Piepers et al., 2007). In short, 10 μL of milk was plated on blood-esculin agar (Oxoid, Erembodegem, Belgium), using 1 plate per cow. Bacteria were identified by colony morphology and Gram staining. For gram-positive cocci, catalase tests were used to differentiate between catalase-positive cocci and catalase-negative cocci. Morphology, hemolysis patterns, deoxyribonucleic acidase testing and pigment production were used to distinguish *Staphylococcus aureus* from the other *Staphylococcus* spp. (Devriese, 1979). *Staphylococcus* spp. other than *Staph. aureus* are referred to hereafter as non-aureus staphylococci. A milk sample was considered culture-positive when growth of ≥ 4 colonies was detected, except for quarters infected with *Staph. aureus* or *Streptococcus agalactiae*, which were considered as infected when growth of ≥ 1 colony was detected. Samples yielding colonies of ≥ 3 different bacterial species were considered contaminated. The plates were incubated aerobically at 37°C and examined at 24 and 48 h. *Corynebacterium bovis* and non-

aureus staphylococci were considered minor pathogens, whereas *Staph. aureus*, esculin-positive streptococci, *Streptococcus dysgalactiae*, and *Strep. agalactiae* were considered major pathogens.

The quarters were allocated to 3 classes based on their IMI status in early lactation according to the result of the bacteriological analyses between 1 and 4 DIM and between 5 and 8 DIM: not infected = no pathogens isolated at both samplings; IMI with a minor pathogen = the same minor pathogen was isolated at the first and second samplings; and IMI with a major pathogen = the same major pathogen was isolated at the first and second samplings. Quarters that could not be allocated to 1 of those 3 classes were considered undefined.

Swabs were streaked onto half of a Petri dish with a blood agar base supplemented with 5% sheep blood (De Vlieghe et al., 2003). The plates were incubated aerobically at 37°C and examined at 24 and 48 h. A teat apex was classified as colonized with non-aureus staphylococci if ≥ 50 cfu were isolated.

Statistical Analyses

Risk Factor Analysis. Prior to statistical analysis, observations were explored and checked for unlikely values. No data were excluded for this reason. The regression-model building process to identify risk factors involved several steps (De Vlieghe et al., 2004a) using MLwiN 2.02 (Centre for Multilevel Modeling, Bristol, UK). Herd, and herd and heifer were included as random effects for the blood and milk analyses, respectively. The continuous independent variables were centered by subtracting their overall mean, providing better interpretations and numerical stability of estimates (Dohoo et al., 2001). Initially, univariable associations were tested between the continuous dependent variables (proportion of induced apoptotic blood PMNL before calving and proportion of induced apoptotic blood PMNL after calving) and all independent variables at the heifer level (Table 1). An identical procedure was followed for proportion of apoptotic milk PMNL after calving and the approximate milk PMNL concentration after calving as dependent variables and all independent variables at the heifer and quarter level (Table 1). Statistical significance in this step was assessed at $P < 0.15$. Second, Pearson and Spearman correlation coefficients were calculated among the significant independent variables to detect multicollinearity associated with each of the 4 dependent variables separately. If 2 independent variables had a correlation coefficient ≥ 0.6 , only 1 was selected for further analysis. Still, none of the variables was excluded for this reason. In the third and final step, multivariable models were fit

Table 2. Herd size, 365-d herd-level milk production, bulk milk SCC, breed, and milk production of the heifers at the first monthly test-day

Item	n	Average	Median	IQR ¹
Herd	19			
Herd size		48	50	37.5–57.5
Herd milk SCC ² (cells/mL)		278,376	271,000	192,500–343,500
365-d milk production (kg)		8,368	8,719	7,677–9,533
Heifer	82			
Breed				
Black and White Holstein-Friesians	27			
Red Holstein-Friesians	33			
Red Holstein-Friesian crossbreds	22			
Milk yield at first monthly test-day (kg)		25.9	25.7	22.9–29.4

¹Interquartile range.²Geometric mean of the herd SCC calculated as the arithmetic mean of the individual cows' SCC on a 4- or 6-weekly basis during 1 yr.

for the 4 dependent variables individually. Statistical significance was assessed at $P < 0.05$. All first-order interactions were tested and removed when nonsignificant (Wald's test $P > 0.05$). To evaluate the proportion of variance occurring at the different levels of the data hierarchy, a 2-level (blood PMNL) and a 3-level (milk PMNL) null model (intercept only) were fit with herd, and herd and heifer, respectively, as random effects. A similar procedure was followed to evaluate the proportion of variance in milk PMNL apoptosis and the approximate milk PMNL concentration after calving at the different levels in a subset of data containing data from the noninfected quarters only. Normal probability plots of residuals and plots of residuals versus predicted values were generated to check whether the assumptions of normality and homogeneity of variance had been fulfilled. No problems were detected.

Blood PMNL Apoptosis Around Calving. The association between the proportion of induced apoptotic blood PMNL before and after calving was tested using mixed models with blood PMNL apoptosis as a dependent variable and the time of measurement (2 levels, before and after calving) as a categorical independent variable. Herd and heifer were included as random effects to account for the clustering of the 2 repeated observations within a heifer and of heifers within herds (MLwiN 2.02; Centre for Multilevel Modeling). Statistical significance was assessed at $P < 0.05$ (Wald's test).

Blood Se. The association between the supplementation of a commercial vitamin-mineral mix before calving (information available per heifer) and the serum Se concentration at approximately 1 wk before the expected calving date of the subset of 60 heifers was calculated using a Mann-Whitney U nonparametric test (SPSS 16.0, SPSS Inc., Chicago, IL). Univariable multilevel regression models including herd, and herd and heifer, respectively, as random effects were used

to evaluate the association between the proportion of induced apoptotic blood PMNL before calving, the proportion of induced apoptotic blood PMNL after calving, and the proportion of apoptotic milk PMNL after calving as continuous dependent variables, and the serum Se concentration before calving as categorical independent variable (low: $<60 \mu\text{g/L}$; high: $\geq 60 \mu\text{g/L}$).

RESULTS

Descriptive Statistics

Average herd size was 48 lactating cows. The average 365-d milk production was 8,368 kg of milk, and the average herd milk SCC was 278,736 cells/mL (Table 2). Thirty-three percent of the heifers were Black and White Holstein-Friesians, 40% were Red Holstein-Friesians, and 27% were Red Holstein-Friesian crossbreds (Table 2).

Thirteen of the 60 heifers from which the serum Se concentration was determined were supplemented with a commercial vitamin-mineral mix. Six of those heifers had a Se concentration $\geq 60 \mu\text{g/L}$ ($n = 13$; 46%), whereas only 7 of the nonsupplemented heifers ($n = 47$; 15%) exceeded that threshold. The serum Se concentration of heifers that were fed a commercial vitamin-mineral mix before calving was 53.8 ± 3.7 (SEM) $\mu\text{g/L}$, whereas that of the nonsupplemented heifers was $35.9 \pm 2.4 \mu\text{g/L}$.

The IMI status in early lactation could be defined for 183 out of 328 quarters. Sixty-three percent ($n = 116$) of those quarters were not infected (class 1), 32.8% ($n = 60$) had an IMI with a minor pathogen (class 2), and 3.8% ($n = 7$) had an IMI with a major pathogen (class 3). Almost half of the teats ($n = 138$, 42.1%) sampled were colonized with non-aureus staphylococci. Heifers were colonized on 2.2 ± 0.1 teats. The percentage of

Table 3. Final multilevel linear models describing the heifer-level risk factors associated with the square-root transformed proportion of apoptotic blood PMNL before and after calving of 82 heifers from 19 dairy herds

Independent variable	n	Apoptotic blood PMNL before calving (%)			Apoptotic blood PMNL after calving (%)		
		β^1	SE ²	P-value	β	SE	P-value
Constant		40.922	2.776	—	41.425	1.827	—
Supplementation minerals/vitamins							NS
No	66	Ref. ³	—	—	—	—	
Yes	16	−10.469	4.843	0.030	—	—	
Season of calving							
January–March	10	0.604	6.596	0.943	2.568	6.474	0.691
April–June	10	Ref.	—	—	Ref.	—	—
July–September	37	−9.258	5.583	0.088	−10.728	5.245	0.041
October–December	25	−18.151	6.076	0.004	−16.445	5.416	0.002
Body condition loss near calving				NS			
<0.25 points	47	—	—		Ref.	—	—
≥0.25 points	35	—	—		7.894	3.336	0.020

¹Linear regression coefficient.²Standard error of the variance estimate of the parameter.³Reference.

non-aureus staphylococci-colonized teat apices per herd varied between 16.7 and 84.2%.

The average proportion of apoptotic blood PMNL at the herd and heifer levels, respectively, was 20.3% [interquartile range (**IQR**): 11.4 to 26.7%] and 19.7% (IQR: 13.4 to 24.6%) before calving, and 20.7% (IQR: 7.7 to 30.3%) and 19.8% (IQR: 8.3 to 26.0%) after calving. The average proportion of apoptotic milk PMNL at the quarter level was 25.0% (IQR: 14.1 to 34.4%). The average approximate milk PMNL concentration was 3.59 ± 0.90 ($\log_{10} \pm$ SD; IQR: 2.89 to 3.09). Considering only the noninfected quarters, the average proportion of apoptotic milk PMNL was 24.6% (IQR: 14.3 to 33.9), whereas the average approximate milk PMNL concentration was 3.33 ± 0.77 (IQR: 2.86 to 3.76).

Risk Factor Analysis

Supplementation of minerals and vitamins before calving was associated with lower proportions of induced apoptotic blood PMNL before calving and with lower proportions of apoptotic milk PMNL after calving (Tables 3 and 4). No such association was present with the proportion of induced apoptotic blood PMNL after calving. The highest proportions of apoptotic blood and milk PMNL were observed in heifers sampled in January to March and April to June, and the lowest in heifers sampled between October and December (Tables 3 and 4). Heifers losing ≥ 0.25 points of their BCS from d 10 before until d 8 after calving had a higher proportion of induced apoptotic blood PMNL after calving (Table 3).

Quarters having teat apices colonized with non-aureus staphylococci before calving had lower proportions of apoptotic milk PMNL and higher approximate

milk PMNL concentrations (Table 4). Quarters that were infected with either a minor or a major pathogen in early lactation had higher approximate milk PMNL concentrations between 1 and 4 DIM compared with noninfected quarters (Table 4). The approximate milk PMNL concentration of heifers with moderate to severe udder edema was higher than that of heifers without or with only a slight degree of udder edema (Table 4). Heifers that had access to pasture for at least the last 2 wk before calving had a lower approximate milk PMNL concentration after calving (Table 4).

None of the 2-way interaction terms was significantly associated either with the blood and milk PMNL viability or with the approximate milk PMNL concentration. The variation in the proportion of induced apoptotic blood PMNL before and after calving resided mainly at the heifer level (Table 5). Both the proportion of apoptotic milk PMNL and the approximate milk PMNL concentration varied mostly at the heifer and quarter levels. Fitting models only including data from the noninfected quarters decreased the total variation in the approximate milk PMNL concentration by 25.0% (Table 5), whereas the variation in the proportion of apoptotic milk PMNL remained virtually unaffected (Table 5). In both models, the variation at the herd level entirely shifted to the heifer and quarter levels (Table 5).

Blood Se Analysis

Heifers orally supplemented with a vitamin-mineral mix before calving had higher serum Se concentration (53.8 ± 3.7 vs. 35.9 ± 2.4 $\mu\text{g/L}$) at approximately 1 wk before the expected calving date compared with nonsupplemented heifers ($P < 0.01$). High serum Se

Table 4. Final multilevel linear models describing risk factors at the heifer and quarter level associated with the approximate milk PMNL concentration and the square-root transformed proportion of apoptotic milk PMNL between 1 and 4 DIM of 82 heifers from 19 dairy herds

Level	Independent variable	n	Apoptotic milk PMNL (%)			Milk PMNL concentration		
			β^1	SE ²	P-value	β	SE	P-value
Heifer	Constant		62.875	2.837	—	33.684	1.616	—
	Supplementation minerals/vitamins							NS
	No	66	Ref. ³	—	—	—	—	
	Yes	16	−6.376	2.501	<0.001	—	—	
	Season of calving							NS
	January–March	10	−0.892	3.997	0.824	—	—	
	April–June	10	Ref.	—	—	—	—	
	July–September	37	−13.187	3.195	<0.001	—	—	
	October–December	25	−13.684	3.337	<0.001	—	—	
	Moderate to severe udder edema before calving				NS			
	No	47	—	—		Ref.	—	
	Yes	35	—	—		4.174	1.612	0.009
Quarter	Access to pasture before calving				NS			
	No	36	—	—		Ref.	—	
	Yes	46	—	—		−3.311	1.616	0.040
	Teat apex colonization with non-aureus staphylococci							
	No	190	Ref.	—	—	Ref.	—	
	Yes	138	−8.648	1.281	<0.001	2.515	1.027	0.014
	Infection status in early lactation ⁴				NS			
	Noninfected	116	—	—		Ref.	—	
	IMI with minor pathogen	60	—	—		2.244	1.140	0.049
	IMI with major pathogen	7	—	—		12.831	3.046	<0.001

¹Linear regression coefficient.²Standard error of the variance estimate of the parameter.³Reference.⁴See text for details.

concentrations were associated with significantly lower proportions of apoptotic blood PMNL before calving compared with low Se concentrations (7.2 ± 0.9 vs. $20.2 \pm 1.7\%$; $P < 0.01$), but no association was found with the proportion of induced apoptotic blood PMNL after calving (19.1 ± 0.3 vs. $15.6 \pm 0.6\%$) or with the proportion of apoptotic milk PMNL (21.9 ± 1.4 vs. $20.2 \pm 1.9\%$).

DISCUSSION

A large proportion of heifers currently calve with one or more quarters with subclinical or clinical mastitis (Fox, 2009). Herd-management practices improved udder health of dairy heifers at calving (De Vlieghe et al., 2004a). Control of heifer mastitis and infectious diseases near calving should include optimization of the general immune status and the immune status of the udder by adjusting management practices.

This study is, to our knowledge, the first to deal with heifer and quarter characteristics associated with the viability of both blood and milk PMNL in periparturient heifers. The availability of an innovative and accurate flow cytometric method (Piepers et al., 2009b) allowed determination of blood and milk PMNL apoptosis of a substantial number of heifers from a substantial number of herds, integrating fundamental research within an

observational setting. Neutrophil apoptosis is one of the most attractive measures to evaluate the first line cell-mediated defense against invading pathogens. Apoptosis of bovine PMNL implies an impaired phagocytotic and oxidative burst activity (Mehrzhad et al., 2004). A reduction in PMNL apoptosis could be beneficial in the elimination of the bacteria in the early stages of IMI as increased numbers of more viable PMNL enhance the defense against the invading bacteria (Dibbert et al., 1999). On the other hand, apoptosis or programmed cell death is a most elegant way of removing PMNL at the site of infection, supporting the resolution of the elicited inflammatory response (Rainard and Riollet, 2006). In this regard, delayed apoptosis may facilitate the ability of bacteria to survive and lead to a persistent accumulation of PMNL, giving cause to chronic infections (Boutet et al., 2004). Although statistically significant associations between blood and milk PMNL apoptosis and several heifer and quarter characteristics were found, this does not prove causality. Rather, some of these associations need confirmation, whereas others generate new hypotheses on improving mammary gland immunity.

A decrease in PMNL functionality was found across the periparturient period of clinically healthy heifers with a low SCC (Mehrzhad et al., 2002). In the present study, in which both healthy heifers and heifers

Table 5. Variance components at each level of the null model and final model for the induced blood PMNL apoptosis before and after calving, and the milk PMNL apoptosis and approximated milk PMNL concentration after calving of 82 heifers from 19 dairy herds

Data set	Data hierarchy	Null model				Final model	
		All quarters		Noninfected quarters		All quarters	
		Var. est. ¹ ± SE ²	% ³	Var. est. ± SE	%	Var. est. ± SE	%
Blood PMNL apoptosis before calving	Herd	88.9 ± 47.6	28.6	—	—	45.7 ± 31.7	18.2
	Heifer	221.2 ± 39.2	71.4	—	—	205.2 ± 36.3	81.8
	Total variance	310.1	100	—	—	250.9	100
Blood PMNL apoptosis after calving	Herd	4.2 ± 21.3	1.6	—	—	22.4 ± 21.1	11.6
	Heifer	252.8 ± 44.3	98.4	—	—	170.3 ± 30.4	88.4
	Total variance	257.0	100	—	—	192.7	100
Milk PMNL apoptosis	Herd	18.3 ± 15.7	8.8	0	0	0	0
	Heifer	95.2 ± 21.2	45.7	94.8 ± 31.0	46.3	57.3 ± 12.5	40.0
	Quarter	94.6 ± 8.6	45.5	110.1 ± 20.4	53.7	85.8 ± 7.8	60.0
	Total variance	208.1	100	204.9	100	143.1	100
Approximate milk PMNL concentration	Herd	4.7 ± 5.2	5.8	0	0	1.7 ± 3.9	2.5
	Heifer	35.3 ± 8.1	43.6	28.3 ± 9.1	47.2	27.6 ± 7.6	41.3
	Quarter	40.9 ± 3.7	50.6	31.6 ± 5.8	52.7	37.6 ± 4.3	56.2
	Total variance	80.9	100	59.9	100	66.9	100

¹Variance estimate.²Standard error of the variance estimate of the parameter.³Variance proportion explained at the different levels.

with subclinical mastitis were enrolled, blood PMNL viability remained stable. A decrease in the proportion of apoptotic blood PMNL because of the presence of IMI in some heifers may have outweighed the increase in the proportion of apoptotic blood PMNL because of the impaired immunity in the periparturient period. This could explain why blood PMNL viability around calving remained more or less unchanged in our study.

Supplementation with minerals and vitamins had a beneficial effect on immune cell activity and the udder health in multiparous cows (Barkema et al., 1998). Our results revealed a beneficial effect of oral supplementation of a commercial vitamin-mineral mix during the last 4 to 8 wk on blood PMNL viability before calving. Selenium contributed to that positive effect. The 2-way interaction term between supplementation with a commercial vitamin-mineral mix and season of calving was not significant, suggesting that the effect of supplementation on blood PMNL viability was not associated with the season of calving and that vitamin-mineral supplementation should be recommended throughout the year. Still, these results should be interpreted with caution as a lack of power could explain nonsignificance of the interaction term.

The threshold for BCS loss was set at a 0.25-unit decrease, because it has been suggested that heifers lose 0.25 points of BCS between 20 d before calving and 7 d after calving (Ruegg and Milton, 1995). Body condition losses of ≥ 0.25 units near calving were associated with

a significantly increased risk for periparturient diseases such as mastitis and metritis in both primiparous and multiparous cows (Ruegg and Milton, 1995), which could be related to the findings in our study.

The seasonal variations in PMNL viability were most likely associated with feeding and pasture management and support results of De Vlieghe et al. (2004a), who found that heifers calving in April to June were at higher risk of having subclinical mastitis in early lactation, possibly because they were kept indoors for a longer period immediately before calving, putting them at increased risk of getting an IMI and saddling them with a lower PMNL viability. Access to pasture for at least the last 2 wk before calving reduced the risk of elevated approximate milk PMNL concentrations in the current study, supporting De Vlieghe et al. (2004a).

Teat apex colonization with *Staphylococcus chromogenes* has a protective effect against IMI in dairy heifers in early lactation (De Vlieghe et al., 2003). Competitive exclusion at the teat apex and the production of inhibitory bacteriocins were proposed as possible protection mechanisms (De Vlieghe et al., 2004b). An increased number of viable PMNL could be another mechanism behind the protective effect of non-aureus staphylococci on the teat apex in general. More research is needed to better understand this finding.

The approximate milk PMNL concentration was calculated indirectly and was only indicative for the absolute number of milk PMNL. Nevertheless, the ap-

proximate milk PMNL concentration was associated with quarter IMI status and was increased more in cases of IMI caused by major pathogens than in those caused by minor pathogens (mainly non-aureus staphylococci), as was expected. Although experimentally induced IMI with *Staph. aureus* and *Escherichia coli* changed the blood or milk PMNL viability of both multiparous cows and heifers (Mehrzhad et al., 2004), no association was found between IMI status and blood or milk PMNL apoptosis in our study. This does not necessarily prove that there is no relationship between PMNL functionality and IMI because many other factors may be involved. The time during the infectious process that the milk samples are collected is probably a major factor (Mehrzhad et al., 2004). The time of onset of the IMI could not be taken into account in the current cross-sectional study, but certainly merits more attention in longitudinal field studies. The 2 samplings for culture in our study were included to improve the accuracy for defining the true infection status of a quarter rather than to conclude whether a quarter was cured or not, especially because false-negative results are not uncommon during routine bacteriologic culturing.

Excessive udder edema is an important risk factor for clinical mastitis in early lactating dairy heifers and cows (Compton et al., 2007). Its association with subclinical mastitis, as indicated by the increased approximate milk PMNL concentration, remains to be demonstrated. Although the causative mechanism of udder edema is not fully understood, the extent of udder edema around calving seems to be mainly affected by diet-related factors (Compton et al., 2007).

The variation in the approximate milk PMNL concentration and the proportion of apoptotic milk PMNL mainly resided at the heifer and quarter level. This observation, together with the shift in variation from the herd to the heifer and quarter levels in a null model only including the noninfected quarters, supports De Vliegher et al. (2004a). Under the current circumstances on Belgian dairy farms, measures to achieve better udder health and to optimize innate immunity should be taken primarily at the heifer and quarter levels rather than at the herd level. Paradoxically, most management practices are applied at the herd level and it remains an everyday challenge for farmers to standardize their management in such a way that all heifers can be kept under optimal conditions. The lack of variation at the herd level may imply that management practices effective in improving innate immunity have not yet been identified and have not been adopted by dairy farmers.

Finally, the high variation in milk PMNL apoptosis among the noninfected quarters within the same heifer is an intriguing observation as initially all milk

PMNL originate from identical blood PMNL having migrated across the blood-milk barrier into the different quarters. A significant variation in the ability of milk PMNL to phagocytose staphylococci among cows and among quarters of the same cow supports this hypothesis (Newbould, 1967). The variation in the approximate milk PMNL concentration at the quarter level remained unexpectedly high as well. The observed variation may be caused in part by false-negative culture results, although this is unlikely because a stringent definition, based on the bacteriologic culture results of 2 consecutive samples, was used to assign quarters as not being infected. Some of the variation in both the proportion of apoptotic milk PMNL and the approximate milk PMNL concentration may be explained by teat apex colonization with non-aureus staphylococci before calving, even though noninfected quarters more likely had their teat apices colonized with non-aureus staphylococci (data not shown) compared with infected quarters.

CONCLUSIONS

Supplementation of minerals and vitamins appears a promising management practice to improve the innate immunity of dairy heifers, certainly given the interrelationship with the serum Se concentrations found. Seasonal variation and factors such as BCS loss and udder edema indirectly suggest that the feeding and pasture management of unbred and pregnant heifers is another interesting area to target. Formulation of path models for blood and milk PMNL viability, including more detailed information related to precalving feeding and variables such as the presence of udder edema at calving and body condition near calving, could provide new insights into the association between individual variables and their effect on innate immunity around calving.

Conducting longitudinal field studies as well as experimental studies would allow the monitoring of the evolution of the blood and milk PMNL apoptosis during infectious processes such as subclinical and clinical mastitis and the effect on bacterial clearance and resolution of the inflammation. The acquired knowledge could help in further validating PMNL apoptosis as a useful indicator of susceptibility to IMI or as a marker for pathogen-specific IMI in the field.

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