Phenotypic and genetic relationships of bovine natural antibodies binding keyhole limpet hemocyanin in plasma and milk

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ABSTRACT

To improve the health status (resilience) of dairy cows, levels of natural antibodies (NAb) might be useful. The objective of the present study was to compare levels and to estimate genetic parameters for NAb measured in milk and plasma samples. Titers of NAb IgM and IgG isotype-binding keyhole limpet hemocyanin of 2,919 cows, in both plasma and milk, were measured using ELISA. Analysis revealed that NAb levels in milk significantly increased with parity, whereas they remained constant in plasma. Moderate positive phenotypic correlations were found between NAb levels in milk and in plasma: 0.18 for IgG and 0.40 for IgM. This indicates that NAb from milk and plasma might reflect different aspects of dairy cow health status. However, high genetic correlations were found for NAb in milk and plasma: 0.81 for IgG and 0.79 for IgM. Heritabilities (SE in parentheses) for NAb measured in plasma [0.15 (0.05) for IgG and 0.25 (0.06) for IgM] were higher than heritabilities of NAb measured in milk [0.08 (0.03) for IgG and 0.23 (0.05) for IgM]. Our results indicate that NAb measured in milk and plasma are heritable and likely have a common genetic background, suggesting that NAb levels measured in milk might be useful for genetic improvement of disease resistance.

Key words: dairy cattle, natural antibody, genetic parameter

INTRODUCTION

Genetic selection for improved disease has become more important because of the limitation of usage of antibiotics. Selection for animal health status is a challenging task due to the lack of reliable, cheap, and easy to measure parameters. Natural antibodies (NAb) might be a novel parameter that enables selection of

cows with an improved ability to stay healthy and to remain productive over a longer period.

In mammals, NAb represent an important component of innate immunity, forming a first line of defense and linking innate and specific immunity (Ochsenbein and Zinkernagel, 2000). Natural antibodies are defined as immunoglobulins derived from self-renewing CD5+ B-1 cells (Casali and Notkins, 1989; Baumgarth et al., 2005). They have been found in all animals tested so far without intentional antigenic stimulation (Avrameas, 1991; Ochsenbein and Zinkernagel, 2000) and have been proposed to reflect the ability of an animal to stay healthy and prolong survival (Boes, 2000; Star et al., 2007).

Natural antibodies are suggested to play an essential role in induction of a primary immune response and improve immune responsiveness to protect individuals against viral and bacterial pathogens (Thornton et al., 1994; Ochsenbein et al., 1999; Kohler et al., 2003; Lammers et al., 2004). In chicken, NAb-binding keyhole limpet hemocyanin (KLH) was indicative for a higher probability of survival during the laying period (Star et al., 2007; Sun et al., 2011). In cattle, NAb were found in both plasma and milk (van Knegsel et al., 2007; Ploegaert et al., 2011) and NAb levels increased with age (Srinivasan et al., 1999; van Knegsel et al., 2007). A positive phenotypic relationship was suggested in dairy cattle between NAb measured in plasma and their energy balance, milk yield, and DMI (van Knegsel et al., 2007). However, in milk, negative relations were found between NAb levels and energy balance, milk yield, and DMI (van Knegsel et al., 2007). Furthermore, Ploegaert (2010) suggested that NAb (especially isotype IgG1) protect against clinical mastitis and high SCC. Heritabilities of KLH-binding NAb levels measured in plasma were 0.18 for IgM and 0.32 for IgG (Thompson-Crispi et al., 2013). Heritabilities of KLH NAb measured in milk were 0.32 for IgG and 0.41 for IgM (Wijga et al., 2013). Together these results show that NAb give information on the health perspective of animals and might also be used for genetic selection to improve health traits.

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Measurement of NAb levels in milk, rather than in plasma, is noninvasive and cheap. Natural antibodies measured in milk may thus be a promising parameter reflecting innate immunity, which might be predictive for disease resistance of dairy cows. As a first step in determining the value of NAb measured in milk as a parameter of disease resistance, phenotypic and genetic relationships of NAb measured in plasma and milk is useful. Knowledge of this relationship is currently lacking. The objective of our study was to estimate phenotypic and genetic parameters for NAb measured in plasma and milk. Genetic parameters will reveal whether NAb levels in plasma and in milk reflect the same trait.

MATERIALS AND METHODS

Animals and Samples

Data used are from the Dutch project WeerbaarVee (resilient livestock). In February and March 2011, plasma and milk samples of 3,034 dairy cows were simultaneously collected from 29 Dutch dairy farms spread over the country. Animals with an unknown ID number or with missing values were excluded from the data set; thus, 2,919 cows were used for the final analyses. To ensure that the results would be representative of future farming conditions, farms were required to have at least 60 cows in milk recording and farms with a mean production level in the lowest quartile were excluded. The farms were chosen from the group of farms with either a high herd age (mean = 4.73 yrs; farm type 1) or an average herd age (mean = 4.16 yrs; farm type 2) in the year before the start of the study. All selected farms needed to participate in the national milk production registration system (MPR) and were required to agree to sampling of plasma and milk from cows and detailed disease recording. Most (72.5%) of the cows included in the study were purebred Holstein Friesians. Furthermore, preliminary results showed no significant effect of breed and it was therefore left out for further analyses. Milk samples were collected in conjunction with MPR from all lactating cows. Plasma samples were taken from about 70 cows per farm: both lactating and nonlactating (dry cows and heifers before calving). The aim of the data set was to keep the distribution of cows over different parities and lactation stage as equal as possible; therefore, cows were selected for plasma sampling based on their parity and lactation stage. Clinically sick animals were not included in MPR and therefore have no milk samples. In total 2,610 milk samples and 2,032 plasma samples were collected. The pedigree file for estimating genetic parameters was

provided by CRV (Cooperative Cattle Improvement Organization, Arnhem, the Netherlands), and included 30,436 animals. Cows used within this study originated from 994 sires and 2,367 dams.

NAb

Natural antibody isotypes IgM and IgG binding *Megathura crenulata*-derived KLH were determined in individual plasma and milk samples by an indirect 2-step ELISA. Flat-bottomed 96-well medium binding plates were coated overnight at 4°C with 1 µg/mL of KLH (MP Biomedicals Inc., Aurora, Ohio), 100 µL/well, in coating buffer (5.3 g/L of Na₂CO₃ and 4.2 g/L of NaHCO₃, pH 9.6). After washing, plasma and milk samples were diluted in 4 steps with a dilution buffer (PBS; 10.26 g/L of Na₂HPO₄·H₂O, 2.36 g/L of KH₂PO₄, and 4.50 g/L of NaCl, pH 7.2, containing 0.05% Tween 20 and 0.5% normal horse serum). Plates were incubated for 1.5 h at room temperature with the samples 1:3 diluted (1:30, 1:90, 1:270, and 1:810). Binding of the antibodies to KLH was visualized using a 1:20,000 diluted rabbit anti-bovine IgGFC labeled with peroxidase (RABo/IgGFC/peroxidase, Nordic, Tilburg, the Netherlands) and 1:20,000 diluted rabbit anti-bovine IgM (RABo/IgM/peroxidase, Nordic). After washing, substrate (tetramethylbenzidine and 0.05% H₂O₂) was added. After 10 min, the reaction was stopped with 2.5 N H₂SO₄. Extinctions were measured with a Multiskan (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Antibody levels (titers) were expressed as log₂ values of the highest dilutions giving an extinction closest to 50% of EMAX, where EMAX represents the highest mean extinction of a standard positive reaction present on each flat-bottomed ELISA-plate (Ploegaert et al., 2010b). The EMAX was calculated based on a plasma-calibrated line for each ELISA plate, for both milk and plasma samples. An amount of 3 titer units was added to all obtained NAb titers to create positive values.

Statistical Analyses

Descriptive statistics were calculated using SAS (version 9.2). Variance components for levels of different NAb isotypes in milk and plasma samples were estimated with a linear animal model:

$$Y_{ijklm} = \mu + \text{PAR}_i + \text{LAC}_j + \text{HERD}_k + \text{animal}_l + e_{ijkl}, \quad [1]$$

where Y was NAb levels (i.e., M-IgG = IgG in milk, M-IgM = IgM in milk, P-IgG = IgG in plasma and P-

Table 1. Numbers, means, and standard deviations (SD) for titers of natural antibody isotypes (IgG and IgM) measured in both milk (M) and plasma (P) samples

Antibody	No.	Mean	SD
M-IgG	2,610	3.25	0.83
M-IgM	2,610	5.60	1.07
P-IgG	2,032	8.03	1.24
P-IgM	2,032	11.56	0.88

IgM = IgM in plasma), μ is the population mean, PAR is fixed effect of parity (i = class of parities, where 1 = heifers before first calving, 2 = parity 1, 3 = parity 2, 4 = parity 3, and 5 = parity ≥ 4), LAC is fixed effect of lactation stage (where 1 = <60 DIM, 2 = 60–120 DIM, 3 = 120–200 DIM, 4 = 200–305 DIM, 5 = >305 DIM, and 6 = nonlactating cows), HERD is random herd effect, animal is random additive genetic effect of the cow, and e is the random residual. Genetic analyses were performed with ASREML software (version 3.0, Gilmour et al., 2009). The estimated variance components from single trait analysis were used to estimate heritabilities. Additionally, phenotypic and additive genetic correlations were calculated from variance components obtained by bivariate analysis.

RESULTS

Descriptive Statistics

Preliminary analysis of the data revealed that all NAb titers were normally distributed. Means and standard deviations of all 4 NAb-binding KLH (IgM and IgG in both plasma and milk) are shown in Table 1. Mean levels of both IgG and IgM in milk were lower

than those detected in plasma. The trait with most variation was IgG in plasma.

In total, 29 farms participated in our study, ranging between 53 and 189 lactating cows per farm, with a mean of 89 lactating cows. Mean herd age of productive cows on farms participating in the study was 4.8 (4.3–6.0) yr. Generally farms classified as high had lower NAb levels than farms classified as average for both NAb levels in milk and plasma. Nevertheless, no significant differences in NAb levels were found between these 2 groups. Farm-type was therefore not included in further analyses.

The cows in our study ($n = 2,919$) had a mean parity of 2.74 (maximum = 14) and were on average 205 DIM (max = 877). Plasma samples were taken from 3 groups: lactating cows, dry cows, and pregnant heifers. Higher NAb levels in milk and blood were detected in dry compared with lactating cows (Table 2). We found that parity significantly ($P < 0.001$) affected NAb levels in milk; overall, NAb levels in milk increased with parity (Table 2). Natural antibody levels in plasma were not significantly influenced by parity (Table 2). The IgM levels in milk significantly decreased after the first lactation stage and significantly increased again toward the end of the lactation period. The same patterns for lactation stages were also observed for IgG, except between the first 3 lactation stages, no significant differences were found. Natural antibody levels in plasma only slightly raised with increasing lactation stage (Table 2).

Genetic and Phenotypic Parameters

Estimates for variance components and heritabilities were obtained from a linear animal model and are

Table 2. Corrected means (LSMEANS) for natural antibody levels (IgG and IgM) in milk (M) and plasma (P) for different parities (PAR class) and different lactation stages (LAC class)

Item	Number	M-IgG	M-IgM	P-IgG	P-IgM
LAC class ¹	1	3.11 ^a	5.39 ^a	7.96 ^a	11.36 ^a
	2	3.04 ^a	5.14 ^b	8.25 ^a	11.61 ^b
	3	3.11 ^a	5.31 ^a	8.17 ^a	11.48 ^{ab}
	4	3.31 ^b	5.81 ^c	8.09 ^a	11.55 ^{ab}
	5	3.59 ^{bc}	6.30 ^d	8.09 ^a	11.62 ^b
	6 (dry cows)	—	—	8.25 ^a	11.99 ^c
PA class ²	1 (heifers before calving)	—	—	8.58 ^a	11.60 ^a
	2	3.29 ^a	5.52 ^a	8.08 ^a	11.56 ^a
	3	3.33 ^{ac}	5.78 ^b	7.93 ^{ab}	11.67 ^a
	4	3.41 ^{bc}	5.99 ^c	7.91 ^{ab}	11.62 ^a
	5	3.45 ^b	5.96 ^c	7.77 ^b	11.51 ^a

^{a–d}Least squares means that do not share a superscript are significantly different ($P < 0.05$).

¹Lactation stage classes 1–6, where 1 = <60 DIM, 2 = 60–120 DIM, 3 = 120–200 DIM, 4 = 200–305 DIM, 5 = >305 DIM, and 6 = nonlactating cows.

²Parity classes 1–5, where 1 = heifers before first calving, 2 = parity 1, 3 = parity 2, 4 = parity 3, and 5 = parity ≥ 4 .

Table 3. Estimates and corresponding standard errors of variance components and heritability of natural antibody isotypes IgG and IgM in milk (M) and plasma (P)¹

Antibody	σ_a^2	σ_h^2	h^2 (SE)
M-IgG	0.05	0.07	0.08 (0.03)
M-IgM	0.22	0.04	0.23 (0.05)
P-IgG	0.22	0.15	0.15 (0.05)
P-IgM	0.19	0.07	0.25 (0.06)

¹Where $h^2 = \sigma_a^2 / \sigma_p^2$, σ_p^2 is the phenotypic variance ($\sigma_a^2 + \sigma_h^2 + \sigma_e^2$), σ_a^2 is the additive genetic variance, σ_e^2 is the environmental variance, and σ_h^2 is the herd variance.

presented in Table 3. Heritability estimates ranged between 0.08 (SE = 0.03) and 0.25 (SE = 0.06). The IgM-binding KLH had higher heritability estimates for both milk and plasma (0.23 and 0.25) compared with IgG-binding KLH (0.08 and 0.15). Heritabilities of NAb levels were higher in plasma for both IgG and IgM (0.15 and 0.25) than in milk (0.08 and 0.23). Positive phenotypic and genetic correlations between NAb levels measured in plasma and milk for both IgG and IgM (Table 4) were observed. Phenotypic correlations between the same isotype in plasma and milk were moderate for both IgM- and IgG-binding KLH (0.40 and 0.18 respectively). Genetic correlations were higher than phenotypic correlations. We found high positive genetic correlations between the same isotypes in plasma and milk (0.79 for IgM and 0.81 for IgG).

DISCUSSION

The aim of our study was to provide greater insight on the levels of NAb isotypes binding KLH collected on the same day in both plasma and milk. A genetic analysis was performed on NAb levels in a large group of cows. Genetic and phenotypic correlations were estimated between NAb levels measured in plasma and milk.

Use of Samples

Different isotypes of NAb can be measured. A large proportion of NAb in circulation are expected to be

of the IgM isotype. Therefore, in our study, the IgM isotype was studied. In addition to IgM, IgG was also chosen, as these 2 isotypes differ from each other in both physiological function and structure. Immunoglobulin G has a monomeric structure, whereas IgM has a pentameric structure. Isotype IgM is polyreactive and more involved in early, innate immune responses, whereas IgG plays a role in the adaptive response (Schroeder and Cavacini, 2010). Therefore analyzing these 2 isotypes is expected to provide more insight in potential differences in NAb levels in plasma and milk. Although IgA can be found in milk, the levels of IgA in plasma were below the detection limit of the assay used for our study, and were therefore not included in present analysis.

In the present study, NAb-binding KLH were used. Other studies on NAb in dairy cows included NAb binding innate antigens such as LPS, lipoteichoic acid, and peptidoglycan (van Kneegsel et al., 2007; Ploegaert et al., 2010b, 2011; Wijga et al., 2013). These other antigens are present on bacteria common in the environment of most dairy cows; KLH, on the other hand, is likely not present in the environment. Exposure to the innate antigens before sampling cannot be excluded, which may complicate comparison of our results with results from other research with other antigens.

Natural antibody levels are influenced by different environmental factors. In the present study, mean NAb levels varied between farms. This is in accordance with earlier studies (Ploegaert et al., 2010b; Wijga et al., 2013). This may be because of housing facilities, farm management, and feeding, or genetic factors resulting from differences in breeding decisions over time.

In the present study, no specific groups of animals were chosen. Within other studies, such as Ploegaert et al. (2011), only cows with low SCC were used; Ploegaert et al. (2010b) and Wijga et al. (2013) only used first-parity cows. Our analysis revealed a clear effect of parity on levels of NAb-binding KLH in milk, which confirms earlier findings in dairy cows by van Kneegsel et al. (2007). This implies that also the age composition of a group of cows should be considered when comparing genetic parameters between studies. This could be explained by the fact that younger ani-

Table 4. Phenotypic (above diagonal) and genetic (below diagonal) correlations (SE in parentheses), between IgG and IgM measured in plasma (P) and milk (M)

Item	M-IgM	M-IgG	P-IgM	P-IgG
M-IgM	—	0.48 (0.06)	0.40 (0.05)	0.16 (0.04)
M-IgG	0.64 (0.17)	—	0.13 (0.04)	0.18 (0.02)
P-IgM	0.79 (0.09)	0.14 (0.23)	—	0.34 (0.04)
P-IgG	0.29 (0.20)	0.81 (0.18)	0.09 (0.20)	—

imals are less exposed to exogenous stimuli than older animals. For example, heritabilities might be lower in a more diverse group of animals, with respect to age, due to more environmental variation. However, other studies in poultry (Berghof et al., 2010) and mammals showed that NAb levels do not only depend on exogenous stimuli, but are also related to autoantigens (Cheng and Chamley, 2008).

Phenotypic Parameters of NAb Levels in Milk and Plasma

The pentameric structure of IgM results in a higher binding (avidity) to antigens than IgG, which has a monomeric structure. Second, IgM is more abundantly present in the body of vertebrates than IgG (Ehrenstein and Notley, 2010). Immunoglobulin M is the first antibody isotype that is produced by B cells. In reaction to antigenic challenge, IgM-producing B-cells can switch to B-cells producing other isotypes under the influence of activated T-cells (Market and Papavasiliou, 2003). In the present study, levels of IgG-binding KLH showed more variation than IgM in plasma (Table 1). The IgG levels are likely to be more affected by environmental exposure to bacteria and other antigens. Wijga et al. (2013) and Thompson-Crispi et al. (2013) found similar results in milk and plasma, respectively.

The present findings showed that NAb levels were lower in milk than in plasma, which is in agreement with earlier studies (Ploegaert et al., 2011; van Kneegsel et al., 2007). The NAb isotypes IgM and IgG may both be transported from plasma to milk (Kacskovics et al., 2000; Östensson and Lun, 2008) in varying degrees depending on stage of lactation, colostrogenesis, and also in response to inflammation in the mammary gland. Phenotypic correlations between NAb levels measured in plasma and milk were moderately positive (Table 4), which means that these correlations are not close to one and are lower than genetic correlations. This could be due to noise in measurements, but also might indicate that NAb measured in milk and plasma might reveal different information regarding the health status of a cow. This finding suggests that phenotypically NAb levels in milk do not perfectly mirror NAb levels in plasma. The positive and high genetic correlations found in our study (Table 4) support a common background of NAb levels in milk and plasma. In an earlier study on 17 cows (different parities and lactation stages, but only cows with a low SCC), a higher positive phenotypic correlation (0.69) was found between the average of 10 individual measurements of total NAb levels binding KLH in milk and plasma (Ploegaert et al., 2011). Using an average rather than single observation reduces the

effect of measurement errors and consequently will lead to higher correlations. In contrast to our results, van Kneegsel et al. (2007) found a small but negative phenotypic correlation between NAb levels in plasma and milk (-0.10). In the latter study, only 76 first-parity cows during early lactation were used. We showed that cows during first stages of lactation have different NAb levels than in later stages of lactation. Such differences in results may rest not only in number of cows but also on distribution over stage of lactation and parity.

Genetic Background of NAb Levels in Milk and Plasma

High positive genetic correlations between level of NAb binding KLH measured in plasma and milk for both IgG and IgM were found (0.79 and 0.81 respectively). This suggests that IgM and IgG measured in plasma and milk are regulated by the same (group of) genes. To our knowledge, no other studies published genetic correlations between NAb levels in plasma and milk. Additionally, we found a moderately positive (0.48) genetic correlation between IgG- and IgM-binding KLH measured in milk. Wijga et al. (2013) found a small but negative genetic correlation between IgM- and IgG-binding KLH (-0.10) in milk. Thompson-Crispi et al. (2013) found a negative genetic correlation (-0.41) between IgM- and IgG-binding KLH in plasma, whereas in the present study a small but positive genetic correlation for both isotypes binding KLH was found.

The present study confirmed that a substantial part of variation between cows of NAb levels binding KLH in both milk and plasma is heritable (Table 3). Heritabilities of IgM- and IgG-binding KLH in milk and plasma were estimated to be between 0.08 and 0.25. Heritabilities of NAb levels in plasma were higher than heritabilities of NAb levels in milk (Table 3). Immunoglobulin M had higher heritabilities for both milk and plasma. This is in agreement with findings of Wijga et al. (2013), who also found the higher heritability (across herd) for IgM (0.41, SE = 0.09) and a lower heritability for IgG-binding KLH (0.32, SE = 0.08) in milk. Higher estimates of heritabilities for IgM might be explained by the fact that IgM is naturally present and more responsible for the first line defense regardless of environmental factors (Kohler et al., 2003; Ehrenstein and Notley, 2010), where IgG is more influenced by environmental factors, which therefore could explain the lower heritabilities for IgG. However, the heritabilities of Wijga et al. (2013) are almost doubled, which can be explained by the use of different data sets. Wijga et al. (2013) used 1,695 first parity cows from 380 farms

sired by 100 bulls. Additionally, only cows off at least 87.5% Holstein Friesian were included. Therefore, cows will have less genetic variation what may cause differences compared with present results. Ploegaert et al. (2010b) found higher heritabilities of NAb-binding KLH in milk [0.36 (across herds) and 0.42 (intra herd)], but no distinction was made between NAb isotypes. Thompson-Crispi et al. (2013) found a higher heritability for IgG (0.32) than the heritability of IgM (0.18), both binding KLH in plasma, which is opposite to current findings. In the study of Thompson-Crispi et al. (2013), however, cows were immunized with hen egg white lysozyme before sampling, which is known to stimulate the antibody-mediated immune response. Therefore, this immunization of cows makes it hard to compare their results to our study, which is directed to naturally present antibodies binding KLH.

From our findings, we can conclude that NAb in both milk and plasma share a common genetic background and show potential for genetic selection. Selection of cows based on NAb levels measured in milk will also lead to correlated response of NAb in plasma. Measurements of NAb levels in milk are less invasive and cheaper than measurements in plasma. Measurement of NAb levels in milk is a more feasible option for genetic evaluation of sires based on a high number of offspring. The value of selection on parameters for NAb depends on its relationship with cow health status and longevity of dairy cows, which is currently being investigated.

CONCLUSIONS

Simultaneously measured NAb levels in both plasma and milk showed high positive genetic correlations for both IgM- and IgG-binding KLH. This indicates that they are regulated by the same set of gene(s). However, phenotypically, NAb levels might give different information on different aspects of a cow's immune status, inferred from to lower phenotypic correlations. Natural antibody isotypes IgG- and IgM-binding KLH in both plasma and milk are heritable, where IgM is more heritable than IgG. Our results indicate that NAb may be valuable for designing breeding strategies for improved health or resilience in cows. Information on NAb as selection tool can be obtained from milk samples, as genotypic correlations with NAb measured in plasma are high. Further studies are in progress to unravel the relations between NAb levels and diseases and to acquire detailed information on reasons for variability in NAb levels influenced by factors such as farm management, genetic background, and functional differences between milk and plasma NAb.

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