Associations between bovine lactoferrin gene polymorphism and somatic cell count in milk

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ABSTRACT: The study included 124 Polish Black-and-White dairy cows of various share of the Holstein-Friesian (HF) breed. Lactoferrin (LTF) gene polymorphism was obtained with PCR-RFLP method using *Eco*RI enzyme. Two alleles of LTF, *A* and *B*, were found in the studied population. Their frequencies were 67.74% and 32.56%, respectively. The alleles controlled the occurrence of three genotypes: *AA*, *BB* and *AB*, of frequencies equal to 37.90%, 2.42% and 59.68%, respectively. It was established that statistically significant associations exist between the somatic cell count (SCC) and LTF genotype, lactation month and parity as well as the HF gene share. No significant association was found between somatic cell count and season. The highest somatic cell count (transformed to a logarithmic scale) was found in milk of the *AB* genotype, whereas the lowest one was found in cows of the *AA* genotype.

Keywords: lactoferrin; polymorphism; somatic cell count; bovine

Inflammatory conditions of the udder (mastitis) represent a major problem in dairy cow management. Producers suffer a huge loss due to veterinary treatment costs or, in some cases, necessary culling of the infected animals. The milk of cows afflicted with mastitis is not suitable for consumption, which also leads to a reduction in the profitability of the production process.

The agents that reduce the incidence of mastitis include lactoferrin. Lactoferrin is an iron-binding glycoprotein found in most exocrine secretions including tears, saliva and milk, and there are numerous reports of its antibacterial activity *in vitro* and *in vivo* (Nuijens et al., 1996; Sordillo et al., 1997; Nibbering et al., 2001). The LTF gene was mapped on chromosome 22q24 (Schwerin et al., 1994; Martin-Burriel et al., 1997). The GenBank presents the sequence of a gene fragment in which a mutation is located. The place of that change is recognized by the restriction enzyme *Eco*RI. At the LFT locus, there were two alleles found, *A* and *B*, which encode three possible genotypes: *AA*, *AB*, and *BB* (Klussmann and Seyfert, 1995; Seyfert and Kuhn, 1994). The frequencies of the alleles were 0.755 and 0.245 for *A* and *B*, respectively (Seyfert and Kuhn, 1994).

Somatic cell count (SCC) in milk constitutes a good diagnostic tool that allows early detection of either subclinical or acute form of mastitis (Green at al. 2004; de Haas at al. 2004), and is therefore a valuable component of monitoring programs (Schukken at al. 2003). SCC is genetically associated with clinical mastitis (r_g = 0.3–0.7) and is more heritable (h^2 = 0.10–0.14) than clinical cases (Mrode at al., 1998).

Besides udder infection, there are a number of other factors influencing somatic cell count, namely lactation number, lactation stage, season or cow genotype (Harmon, 1994). Molecular genetic markers were also associated with changes in SCC (Ashwell et al., 1997; Zhang et al., 1998; Klungland et al., 2001).

Taking into account the facts mentioned above, it is reasonable to investigate in pursuit of any associations between LTF polymorphism and somatic cell count (susceptibility/resistance to mastitis).

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MATERIAL AND METHODS

The study included a herd of 124 dairy Polish Black-and-White cows of various gene share (44–94%) of the Holstein-Fresian (HF) breed. The studied herd was kept on a farm located in the Pomerania region, in the north-western part of Poland. All animals were kept in identical environmental conditions. They were fed standard feed rations and seasonally (in spring and summer) were put out to pasture. The cows were milked twice a day with the use of a pipeline milking machine. The herd's milk yield was evaluated with A4 method in compliance with the recommendations of the International Committee for Animal Recording (ICAR). The data concerning somatic cell count in milk were collected in 2002 on the basis of monthly milking tests, representatively sampled from both of the two milkings (992 samples) performed at the same day time for each cow. SCCs in the samples were determined with an instrumental method in compliance with the PN-EN ISO/IEC 17025 standard, using Combifoss equipment (including Fossmatic 5000 apparatus, Foss, Hillerod, Denmark). Analyses were carried out in the ICAR and COFRAC certified laboratory of milk analyses in Krotoszyn, Poland.

Peripheral blood to be used for DNA isolation was collected from all the cows and placed in test tubes containing EDTA as an anticoagulant. The isolation of DNA from the whole blood sample was performed with the method described by Kanai et al. (1994).

The isolated DNA was used for PCR amplification of the LFT gene fragment of 301 bp (base pair) with the use of the following primers:
Forward: 5'-GCC TCA TGA CAA CTC CCA CAC-3'
Reverse: 5'-CAG GTT GAC ACA TCG GTT GAC-3'

The PCR was performed according to Seyfert and Kuhn (1994). Restriction analysis of the amplified fragment was done with RFLP using *Eco*RI enzyme (for 3 h, with 5 units/20 ml, at 65°C) and restriction fragments were analysed electrophoretically in 2% agarose gel in TBE buffer. The *Eco*RI digestion produced a mixture containing fragments of 301 bp (allele *A*, no sequence recognized by the restriction enzyme), 201 bp, and 100 bp (allele *B*).

The frequencies of LTF alleles and genotypes were determined and it was verified with χ^2-test whether their distributions conformed to those expected (according to the Hardy-Weinberg law). The statistical analysis also included research for associations between LTF polymorphism and SCC in milk. Holstein-Friesian gene share, parity, season, month of lactation (lactation stage) and cow (random factor nested in LTF genotype) were also treated as sources of variability. The year was divided into two seasons: autumn/winter – from October to March, and summer/spring – from April to September. Lactation number 5 and the later ones were treated as one category. SCC was transformed to a logarithmic (log2) scale in order to balance the distribution. The following statistical model was applied:

$$(\text{log2 SCC})\ y_{ijklm} = \mu + a_i + b_j + c_k + d_l + f_m(a_i) + g(\text{HF}) + e_{ijklm}$$

where:

y_{ijklm} = somatic cell count (log2 SCC)
μ = mean somatic cell count for herd (log2 SCC)
a_i = effect of LTF genotype
b_j = effect of lactation number
c_k = effect of lactation month (lactation stage)
d_l = effect of season
$f_m(a_i)$ = effect of cow, random factor nested in LTF genotype
g (HF) = coefficient of regression of HF gene share on SCC in milk
e_{ijklm} = error

The results of the analyses were processed statistically according to Statistica data analysis software system, version 6.0 (StatSoft, 2001), with GLM multiple-factor, mixed, nested model. The correlation coefficient between SCC and HF gene share was also calculated.

RESULTS

Two alleles of LTF, *A* and *B*, were found in the studied population of dairy cows. Their frequencies were 67.74% and 32.56%, respectively. The alleles controlled the occurrence of three genotypes – *AA*, *BB* and *AB*, with their frequencies of 37.90%, 2.42% and 59.68%, respectively (Table 1). Statistically significant (P = 0.000252) deviations were found in the analysed population between the observed distribution of LTF genotypes and their expected distribution estimated according to the Hardy-Weinberg law. Significantly more heterozygous *AB* genotypes were found in relation to the expected rate of heterozygotes, whereas there were significantly fewer *BB* homozygotes in comparison

Table 1. Frequencies of LTF genotypes in the analysed population

LTF genotype	Observed frequency (%)	Expected frequency (%)	Chi-square
AA	37.90	45.89	1.72364
BB	2.42	10.41	7.60562
AB	59.68	43.70	7.24358
Total	100.00	100.00	16.57284

Chi-square = 16.57284; $df = 2$; $P \leq 0.000252$

Table 2. Associations between log2 SCC and the analysed factors

Source of variability (analysed factors)	Degrees of freedom	Statistic F	Probability P	Significance of associations
Regression for HF genes share	1	5.856	0.016	*
1. LTF genotype	2	3.369	0.036	*
2. lactation number	4	4.510	0.001	***
3. lactation month	12	2.470	0.004	**
4.season of year	1	0.301	0.584	n.s.
5. cow nested in LTF genotype	120	3.511	0.000	***

*$P \leq 0.05$; **$P \leq 0.01$;***$P \leq 0{,}001$; n.s. = non significant

with those expected. The disturbance in the genetic balance of the population may have resulted from unintended selection for this trait, coupled with the selection for performance.

The study searched for associations between LTF genotypes and SCC in milk, taking into account such factors as lactation parity, month of lactation, season, cow and Holstein-Friesian gene share. The results are presented in Table 2.

It was established that statistically significant associations exist between SCC and LTF genotype, lactation parity, month of lactation, cow and HF gene share. On the other hand, no significant relationship was found between SCC count and season (Table 2).

The highest SCC count (transformed to a logarithmic scale) was found in the milk of the cows with *AB* genotype, while the lowest were in the cows with *AA* genotype (Table 3). Furthermore, a high, statistically significant association was confirmed between SCC and parity. The highest SCC was found in the milk of cows in the 1st and 2nd as well as 5th and higher lactation number. It was also found that SCC was generally lower in the initial months of lactation (except for the first month), and grew in the subsequent months (especially in a dry period). A positive, significant association was also established between SCC and HF gene share. The correlation coefficient between HF gene share and SCC was not high (0.106) but statistically significant ($P \leq 0.02$). The animals with a higher share of HF genes were found to have a higher level of somatic cells in their milk. This can indicate a lower resistance of the HF cattle to mastitis.

DISCUSSION

Seyfert and Kuhn (1994) found two alleles, *A* and *B*, in the LTF locus, which encoded three possible genotypes: *AA*, *AB*, and *BB*. The frequencies of the alleles were 0.755 and 0.245 for *A* and *B* respectively, thus the results were similar, with a slightly higher frequency of the allele *A*.

Lactoferrin is involved particularly in the mechanism of alimentary immunity (Rainard, 1986, 1987; Schutz et al., 1994; Seyfert et al., 1997; Kanyshkova et al., 2001). This immunity results from the fact that possible infection factors have a limited availability of iron (as well as other growth agents,

Table 3. Means and standard deviations of SCC (log2 SCC) in milk in relation to analysed factors

Effect	Number of samples	Mean of log2 SCC	Standard deviation
LTF *AA*	395	6.97	1.70
LTF *AB*	553	7.44	1.63
LTF *BB*	44	7.13	2.20
Lactation I	126	7.24	1.08
Lactation II	435	7.25	1.80
Lactation III	259	6.97	1.61
Lactation IV	59	8.54	1.80
Lactation V and higher	113	7.13	1.73
1. month of lactation	61	7.19	2.31
2. month of lactation	71	6.35	2.10
3. month of lactation	80	6.63	1.86
4. month of lactation	84	6.85	1.20
5. month of lactation	82	6.92	1.68
6. month of lactation	101	7.30	1.72
7. month of lactation	99	7.25	1.51
8. month of lactation	113	7.53	1.52
9. month of lactation	95	7.45	1.06
10. month of lactation	82	7.60	1.33
11. month of lactation	67	7.78	1.22
12. month of lactation	36	7.78	1.19
13. month of lactation	21	7.28	1.25
Autumn/winter	347	7.20	1.67
Spring/summer	645	7.26	1.71
Total	992	7.24	1.70

such as phosphorus and zinc), since its concentration in an organisms fluids is reduced (Rainard, 1987; Carlsson et al., 1989; Persson, et al., 1992). Another function of lactoferrin is to inhibit enteric absorption of iron in neonates. Lactoferrin may also take part in intracellular destruction of bacteria performed by inducing hydroxyl radical formation, which is catalyzed by iron (Fang and Oliver, 1999). The fact that lactoferrin appears in infected areas also due to its local synthesis (Senft and Neudecker, 1991; Persson et al., 1992). For example, an infection results in 30-fold increase in the synthesis of the protein in secretory cells of the mammary gland (Kawai et al., 1999). The concentration of lactoferrin in normal bovine or murine milk is reported to be between 20 and 200 μg/ml (Neville et al., 1998). In addition, LTF stimulates the immune system, and serves as a natural antioxidant (Detilleux, 2002). Lactoferrin may be active in modulation and regulation of macrophages, lymphocytes and neutrophil function (Smith and Oliver, 1981; Sordillo et al., 1997). Due to its properties, lactoferrin is one of the more important factors that prevent and control mastitis in dairy cows (Klussmann et al., 1996; Seyfert et al., 1996; Hirvonen et al., 1999; Klungland at al., 2001; Teng, 2002).

Furthermore, SCC generally increases with advancing age and stage of lactation. An effect of lactation number, lactation stage and breed was reported by Schutz et al. (1994) and Cameron and Anderson (1993). Similar associations between SCC in milk and lactation number (age), herd, breed and lactation stage (days elapsed from calving) were published by a number of authors (Laevens et al., 1997; Busato et al., 2000). Sheldrake et al. (1983) confirmed that milk from uninfected quarters dis-

plays little change in SCC as number of lactations increases. Nikodemusz et al. (1994) established that maximum SCC in the milk of HF and Hungarian Red-Spotted cows fell in the first month of lactation. In the second month, SCC remained high, and afterwards decreased in subsequent months to grow again from the 7th month on.

SCC is generally lowest during the winter and highest during the summer (Dohoo and Meek, 1982), which coincides with an increased incidence of clinical mastitis during the summer months (Smith et al. 1985). Smith et al. (1985) showed that the rate of infection with environmental pathogens was highest during the summer, and coincided with the highest number of coliforms in bedding. They suggested that the stress of high temperatures and humidity could have increased susceptibility to infection as well as increased the number of pathogens to which the cows were exposed. These findings support the concept that temperature stress *per se* is not the cause of increased SCC, but the increased SCC is a result of greater exposure of teat ends to pathogens, resulting in more new infections and clinical cases during the summer months.

Furthermore, Norman et al. (2000) studied the relationship between SCC and climatic conditions in the USA. The SCC was lower in the western states and higher in the south-eastern states. SCC was also lower during autumn and winter (from October to January) and higher during summer (from July to August).

CONCLUSION

The results obtained in this study confirmed the hypothesis that LTF gene can be used as a marker of somatic cell concentration in milk and, in consequence, as a marker of susceptibility/resistance to mastitis in dairy cows. Additional studies on this problem, however, are necessary to confirm associations between lactoferrin genotype and SCC before this criterion is used in large-scale selection.

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