

Bovine lactoferrin gene polymorphism applicability in the selection of cows resistant to mastitis*

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Lactoferrin gene polymorphism was analysed in order to test its usefulness for marker-assisted selection of cows resistant to mastitis. For this purpose a total of 698 blood samples obtained from Polish Holstein-Friesian Black-and-White cows were examined. DNA was extracted from blood leukocytes and used for lactoferrin gene polymorphism analysis conducted by the PCR – RFLP method. Statistical analysis was performed with the use of the GLM procedure (SAS software). Effects of lactation, days in milk, milk yield on the milk recording day, herd and season of the year were included in the statistical model for associations between genotype and somatic cell count. Cows carrying allele B of the lactoferrin gene were more resistant to mastitis than the other animals and had a lower somatic cell count in milk. Application of lactoferrin gene variants as genetic markers for mastitis can facilitate selection of cows less prone to mammary gland infections.

KEY WORDS: dairy cattle / mastitis / lactoferrin / polymorphism

In view of the economic importance of the problem various research teams worldwide have been trying to find not only an easily applicable, but first of all an efficient method to prevent mastitis. To date it has not been attained either using conventional treatment methods or hygienic milking technologies. Procedures aiming at the limitation of udder inflammation incidence in dairy cattle are time-consuming and costly, while at the same being rather ineffective.

Due to the confirmed genetic antagonism between milk yield and the health condition of the udder, resulting in the deterioration of the udder health status as a consequence of selection towards increased milk yields, the situation has become progressively worse.

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Susceptibility or resistance to mastitis in dairy cattle has the genetic background (thus it may be inherited) and depends on multiple genes. However, it is a character of low heritability, which to a considerable degree hinders traditional selection of cows based on phenotype. Additionally, this selection is limited due to the weakly defined traits (as it is difficult to define and measure resistance) and problems with the application of data concerning the incidence of clinical cases of mastitis (a problem observed in most countries, including Poland).

At present the primary task for researchers is to search for a reliable tool, which would improve the udder health status in cows while at the same time maintaining high milk production, which is a necessary pre-condition for profitability of dairy cattle breeding. Good prospects for an improvement in the udder health status are provided by marker-assisted selection (MAS). The MAS approach uses genetic markers to select animals which are less susceptible to udder inflammations [9, 11, 13]. In the case of quantitative traits, characteristics which are weakly defined, related to reproduction or manifested only in one sex (such as mastitis occurring only in females), this approach provides a theoretical chance to attain the assumed genetic objective within a short time and at low financial costs. Over a longer timeframe the application of MAS could facilitate the elimination of an undesirable effect of conventional selection, i.e. reduced genetic variation resulting from the limitation of the allele pool in the population. Probably in the future it will be possible to characterise an animal in terms of many different alleles, which will facilitate the preservation of genetic variability of animals while simultaneously ensuring a large production scale.

The aim of the study was to determine the applicability of the lactoferrin gene polymorphism in the selection of cows resistant to mastitis.

Material and Methods

Analyses within this study were conducted in a herd of 698 Polish Holstein-Friesian Black-and-White cows. The animals were kept on a dairy cattle farm in the Lubuskie province, Poland. All the animals were kept under identical housing and feeding conditions.

Material for DNA isolation consisted of peripheral blood collected from the jugular vein. Blood samples of 7.5 ml were collected to vacuum test tubes containing 1.6 mg anticoagulant (K-EDTA). DNA was isolated following the methodology presented by Kanai et al. [4]. Isolated DNA was stored at -20°C until analyses.

In this study it was decided to perform the lactoferrin gene fragment amplification using touchdown PCR. This method is a variant of the PCR technique, in which changes in annealing temperature prevent the appearance of large amounts of undesirable strand segments in the reaction product. Such segments may otherwise appear during the reaction as a consequence of faulty primer activity [2]. The PCR product was an amplified DNA fragment of 301 base pairs (bp) in length, which was subjected to the action of the *EcoRI* restriction enzyme at a temperature of 37°C for 3 hours. The electrophoretic separation was run on 2% agarose gel in the TBE buffer with an addition of ethidium bromide in the presence of the pUC19/*MspI* DNA template (Fermentas) at a constant voltage of 100 V.

The restriction fragments of 200 and 101 bp were identified under UV light and recorded in the database.

In order to determine the genetic structure of the analysed herd of cows based on the lactoferrin gene polymorphism the following parameters were estimated:

- frequency of individual alleles and genotypes;
- the polymorphic index and heterozygosity;
- consistency with the Hardy-Weinberg equilibrium (HWE).

The database comprised records concerning each animal such as their identification number, date of calving and milk recording date, pedigree, results of milk performance testing (daily milk yield, percentage and yield of fat and protein in milk) and somatic cell count.

Statistical analysis concerning the effect of tested factors on the somatic cell count was performed using the GLM procedure in the SAS software package. The analysis of variance was applied to assess the dependence between the somatic cell count (SCC) and the lactoferrin gene genotype. In view of a lack of normal distribution in the case of SCC the common logarithm was prepared for this variable (LSCC).

The following statistical model was applied:

$$Y_{ijkl} = \mu + A_i + E_j + L_k + \beta(x_{ijkl} - \bar{x}) + e_{ijkl}$$

where:

Y_{ijkl} – logarithm of somatic cell count,

μ – population mean,

A_i – the effect of lactoferrin gene genotype ($i = 1, 2, 3$),

E_j – the effect of the year and season of analysis ($j = 1, \dots, 23$),

L_k – the effect of lactation ($k = 1, \dots, 6$),

$\beta(x_{ijkl} - \bar{x})$ – regression for milk yield,

e_{ijkl} – random error.

Results and Discussion

In the investigated population the average milk yield in a 305-day lactation was 9662 kg, the yield of fat was 367 kg (3.85%), while that of protein – 323 kg (3.36%), respectively (Table 1). The average somatic cell count in milk was 531 thousand/ml. The dry period for the cows lasted on average 55 days. The age of first calving of heifers was 794 days, the calving interval was 450 days, while the interpregnancy period was 181 days. Pregnancy typically lasted 279 days, while complete lactation was 342 days. The average productive life of cows in the herd was 894 days. The breeding window was 88 days, whereas anoestrus lasted 112 days. Analysis of culling causes showed that the highest percentage of culling cases was recorded in the 4th and successive lactations (18.3). It needs to be stressed that after the 1st lactation as many as 17.4% primiparous cows were eliminated from the herd. Sterility was the most frequent cause for culling (49.9%), followed by udder diseases (16.7%). Culling caused by low productivity accounted for 9.3% all cases.

Table 1
Milk yield of the tested dairy herd

Trait	\bar{x}	Sd
Milk yield – 305 days (kg)	9662	1947
Fat content (%)	3.85	0.36
Protein content (kg)	3.36	0.20

In the analysed herd 3 lactoferrin genotypes were found with the following frequencies: *AA* – 0.54, *AB* – 0.38 and *BB* – 0.08 (Table 2). The incidence of these genotypes was determined by the presence of two alleles, with the following restriction fragment lengths observed on agarose gel: allele A – 301 bp and allele B – 200 and 101 bp. Genotype *AA* of the lactoferrin gene was recorded in a half of the investigated cow population, while the frequency of genotype *BB* was very low, as it was observed in approx. 8% analysed animals. In an earlier study by Sender et al. [10, 12, 14] it was stated that cows with the *BB* lactoferrin genotype are least susceptible to mastitis. Among the animals included in this study, similarly as in the previously analysed population, cows with the *BB* lactoferrin genotype were relatively rare. The low frequency of the *BB* genotype in the population of dairy cows may limit the chance for statistical confirmation of a linkage of allele B with reduced susceptibility of cows to mastitis. The low frequency of this allele may be caused by its simultaneous linkage with low milk yield in cows. The gradual elimination of allele B during long-term selection towards high milk yield is likely.

Table 2
Frequency of lactoferrin genotypes

Genotype	Number of cows	Frequency
<i>AA</i>	257	0.54
<i>AB</i>	181	0.38
<i>BB</i>	37	0.08

The frequencies of individual lactoferrin genotypes in the analysed population are comparable to those recorded by Wojdak-Maksymiec et al. [16]. Those authors reported the frequency of the *AA* genotype at 0.379, *BB* – at 0.0242 and that of *AB* at 0.5968, i.e. a greater number of heterozygotes in relation to the *AA* homozygotes than it was in this study.

Table 3 presents frequencies of the lactoferrin gene alleles, which were recorded in the investigated population. The frequency of allele B is approx. 3.81-fold lower than allele A. In research conducted to date on polymorphism in intron 6 a similar frequency

Table 3

Frequency of lactoferrin alleles in the examined population and Hardy-Weinberg test results

Allele	Frequency	Chi ²	Degrees of freedom	Probability	
				Chi-test	expected value
A	0.732	0.42	1	0.5175	0.4931
B	0.268				

of lactoferrin gene alleles was recorded [11, 12, 15, 16]. In this study statistically significant differences were recorded between the expected distribution of alleles in the analysed population and the actual distribution (Table 3). In this population the lack of consistency with the Hardy-Weinberg law may have been caused by the long-term selection targeting productivity traits.

Table 4 presents results concerning the relationship of the lactoferrin genotype with the somatic cell count, indicating the udder health status of these cows. The highest somatic cell count was recorded in cows with the *AA* lactoferrin genotype, being significantly higher ($p < 0.05$) than in milk of heterozygotes. No statistically significant differences ($p < 0.07$) were recorded between the *BB* and *AB* genotypes. In the *AB* heterozygotes and the *BB* homozygotes the somatic cell counts were lower.

Table 4

Somatic cell count (logarithmic value) depending on lactoferrin genotype

Lactoferrin genotype	Number of samples	LSM	Se
<i>AA</i>	7854	5.71 ^a	0.03
<i>AB</i>	5854	5.65 ^a	0.03
<i>BB</i>	1064	5.62	0.05

LSM – Least Square Mean

Se – standard error

a – statistically significant differences at $p < 0.05$

Based on the presented results cows with the *BB* and *AB* genotypes should be recommended for breeding, as those characterised by better udder health. Unfortunately, as it was mentioned earlier cows with the *BB* genotype are relatively rare, in the investigated population there were only 37 such cows, i.e. 8% of the total population.

A slightly different trend was reported in earlier studies, in which the highest mean LSCC was associated with the *AB* lactoferrin genotype, while the *BB* genotype was linked with the lowest LSCC [12]. In a study by Wojdak-Maksymiec et al. [16] the lowest mean LSCC was recorded for animals with the *AA* lactoferrin genotype, followed by the *BB*

genotype, whereas the highest LSCC was found in animals with the *AB* genotype. It needs to be stressed that previous studies concerning polymorphism in intron 6 of the lactoferrin gene were based on a smaller number of animals than this study.

Apart from the analysed polymorphism, observed in intron 6, there are other polymorphic forms of the lactoferrin gene. To date polymorphism of this gene has been found both in the regulatory region and within coding sequences and introns of this gene [3, 5, 6, 15]. Gene polymorphisms detected in the promoter probably affect its expression. It was found that polymorphism in position +32 (C/G) has a significant effect on the level of protein and its percentage content in milk, while it does not significantly influence the somatic cell count [3]. Kamiński et al. [3] suggested that allele G reduces lactoferrin expression, which as a consequence is manifested in a lower somatic cell count, while allele C increases lactoferrin expression, resulting in an enhanced immune response and an increase in somatic cell count in milk.

The recently presented results of Bahar et al. [1] show that polymorphisms located within the promoter region of the lactoferrin gene influence contents of this protein in milk.

A highly important role played by lactoferrin is connected with its interaction with lysozyme in the destruction of bacterial cell walls, which may enhance the sensitivity of bacteria to certain antibiotics. In some cases this facilitates a 2-fold reduction of the therapeutic concentration of the antibiotic. Normal cow's milk contains slight amounts of lactoferrin, max. around 0.1 mg in 1 ml. Lactoferrin concentration in the secretion from dried udders is many times greater and amounts to approx. 20 mg/ml, and frequently even more. In milk of cows suffering from mastitis the concentration of lactoferrin is greater than in milk from healthy cows, as it amounts to approx. 0.3-2.3 mg/ml.

In the case of weakly defined traits, including also resistance to mastitis, it is difficult to indicate candidate genes controlling the variability of traits, since it is dependent on many factors (physiological mechanisms, pathogens, etc.). Despite difficulties, recently many efforts have been made to identify genetic markers for mastitis [7, 8].

Results of this study indicate a potential applicability of the bovine lactoferrin gene as a candidate gene or a marker for cows' susceptibility to mastitis; however, it is necessary to search for a relationship of other polymorphic forms of this gene with the incidence of mastitis.

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