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L-Selectin Gene Polymorphism and Its Association with Clinical Mastitis, Somatic Cell Score, and Milk Production in Polish Holstein-Friesian Cattle

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ABSTRACT

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Bovine mastitis is a widespread disease of the mammary gland, highly contributing to the increase in veterinary costs in dairy industry. In the present study, the genetic polymorphism within bovine *L-selectin* gene was analysed and its impact on clinical mastitis occurrence, somatic cell score (SCS), and milk production traits in Polish Holstein-Friesian cows was examined. Polymorphism within *L-selectin* gene, molecule responsible for neutrophil attachment to endothelium, might have a potential role in immune response to bacterial infections and udder health. Two hundred and six Polish Holstein-Friesian cows were genotyped by polymerase chain reaction-restriction fragment length polymorphism method. Two single nucleotide polymorphisms mutations within the coding sequence of *L-selectin* gene were identified (c.165G>A and c.567C>T). The effect of c.165G>A and c.567C>T mutations on SCS was highly significant (P = 0.0019 and P = 0.0003, respectively). Strong associations ($P \leq 0.0001$) were also observed between *L-selectin* polymorphism and milk production traits (milk yield, milk fat percentage, and milk protein percentage). However, the polymorphism in the analysed gene had no influence on the resistance or susceptibility of cows to clinical mastitis (only the tendency toward significance, P = 0.06 for c.567C>T mutation was found). Potential exploitation of the information on the identified associations in genetic selection needs to confirm the obtained results in further investigations.

Keywords: *SELL*; udder; inflammation; milk production traits; cows

Mastitis, the inflammation of the mammary gland, is nowadays a serious problem in dairy farming. It affects many high producing cows, being one of the most prevalent and costly diseases (Petrovski et al. 2006). Moreover, mastitis contributes to the compromised quality of milk and dairy products (Thompson-Crispi et al. 2014).

Despite the worldwide efforts to improve herd management practices, mastitis control in dairy industry remains inadequate. Thus, there is a

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high demand for measures which would allow to reduce the incidence of this disease. Since mastitis is characterized by pathological changes in the udder tissues in consequence of bacterial infections, the use of antibiotics is one of the treatment options. However, this solution is not optimal, given that there is a general endeavour to restrain the use of antibiotics with the purpose of limiting the emergence of antimicrobial resistance (Mallard et al. 2015). Another option can be breeding for more robust cows. For many years, livestock species have been mainly selected for production traits. Until recently, less attention has been paid to health traits, including immune response. According to Heringstad et al. (2003), genetic improvement of mastitis resistance can be achieved by traditional breeding. However, it is also known that this trait is lowly heritable and unfavourably correlated with milk production traits (Heringstad et al. 2000; Negussie et al. 2008). Therefore, breaking negative genetic correlation between these traits and subsequent improving all of them simultaneously needs understanding of genetic mechanisms which are involved both in the resistance to mastitis and in milk production (Olsen et al. 2016).

Leukocytes recruited from the peripheral blood to milk are a part of the inflammatory response to invading bacteria during mastitis. Those playing a critical role in the host defense against bacterial infections are neutrophils and macrophages. It has been shown that weak recruitment of neutrophils corresponds to the increased severity of the inflammation (Hill et al. 1979; Van Werven et al. 1997). Because of the ability to phagocytose and kill bacteria, the most numerous circulating blood leukocytes, polymorphonuclear neutrophils (PMN), provide the first line of defense against infection (Prin-Mathieu et al. 2002; Paape et al. 2003). On the PMN surface, many receptors and adhesion molecules are present. They comprise, for example, G-protein-coupled chemokine and chemoattractant receptors, cytokine receptors, innate immune receptors such as Toll-like receptors and C-type lectins, Fc-receptors, and adhesion receptors such as selectins and integrins (Futosi et al. 2013).

One of the most important adhesion molecules expressed on bovine PMN is L-selectin, coded by the *SELL* gene, named also CD62L, which is responsible for neutrophil attachment to endothelium (Kishimoto and Rothlein 1994). L-selectin mediates the migration of activated circulating PMN across the blood–milk barrier in the process of diapedesis through the endothelium of the mammary gland (Diez-Fraile et al. 2003). It was proved that inhibiting or modifying the L-selectin expression on neutrophils has negative effect on progression of the inflammatory response during bacterial infection (Weber et al. 2004). Given the importance of L-selectin for modulating inflammation and the host immune response, single nucleotide polymorphism (SNP) within this gene may be suitable marker for disease resistance in dairy cattle.

The variants in genes encoding molecules of immune system have been previously associated not only with udder health, but also with milk traits, including milk yield and milk protein yield (Leyva-Baca et al. 2007; Pant et al. 2007). Thus, it was considered reasonable to investigate the associations between *L-selectin* polymorphism and milk traits in this study.

We hypothesized that SNPs in the *SELL* gene contribute to variation in health and production traits in dairy cattle, so the aim of our study was to investigate possible associations between the mentioned polymorphisms and udder health and milk production traits in Polish Holstein-Friesian cows.

MATERIAL AND METHODS

The study was performed on 206 Polish Holstein-Friesian cows, being the daughters of 97 sires. All cows were maintained at one farm in south-western Poland, in identical environmental conditions and were fed with the Total Mixed Ration (TMR) system. They had completed at least the first three lactations.

Genomic DNA was isolated from the cow's milk using an original method developed in the authors' laboratory (Pokorska et al. 2016). Total DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and samples with optical density ratio at A260/A280 between 1.8 and 2.0 were utilized for this study.

Genotypes of the cows were determined with the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Primers for PCR (Table 1) were designed based on

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the reference sequence (GenBank Accession No. NM_174182.1). *L-selectin* gene contains 9 exons; in this study sequences of exons 3–6 were covered.

The PCR for each exon was performed in a C1000 Thermal Cycler (Bio-Rad Laboratories, Inc., USA), in a final volume of 20 µl, containing approximately 150 ng template DNA, 2 µl 10× PCR buffer, 2.25 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM forward primer, 0.3 µM reverse primer, and 1.75 U *Taq* polymerase. All reagents were supplied by Thermo Fisher Scientific (USA). The PCR program included the following steps: initial denaturation at 95°C for 5 min, followed by 34 amplification cycles (each cycle included: denaturation at 95°C for 45 s, annealing of primers at 63°C for 40 s, and elongation at 72°C for 50 s) and final elongation at 72°C for 6 min.

The amplified products for sample cows were sequenced in a commercial laboratory (Genomed S.A., Poland) by Sanger method. Sequences of *L-selectin* gene of sample cows were compared with the GenBank reference sequence and with each other to identify SNPs. For preliminarily identified polymorphisms, discriminating restriction enzymes *Bsm*I and *Apa*I were selected. The amplified products were digested with restriction enzymes as recommended by the manufacturer (Thermo Scientific). The obtained restriction fragments were separated in 3% agarose gel stained with SYBR Safe dye (Invitrogen/Thermo Fisher Scientific).

Genotypic and allelic frequencies were calculated using the FREQ procedure of SAS software (Statistical Analysis System, Version 9.2). The Hardy-Weinberg equilibrium of the mutation was determined by chi-square test. Prediction of *L-selectin* haplotypes in the herd was performed using HAPLOVIEW software (Barrett et al. 2005).

Table 1. Amplification primers for analysed exons of bovine *L-selectin* gene

Exon No.	Primer sequence	Product size (bp)
3	F: 5'TGCAGCTACACAATTCACACTG 3' R: 5'CAGGTTCCCATGGGGTTAG3'	499
4	F: 5'AAAATAGTCACAGCCAAAAAGTTG 3' R: 5'TATCATTTTCCCCCAACCAA 3'	281
5	F: 5'AAATTCAAACCCGGTCATCA3' R: 5'TCCAGGATTCCCACGATAAA 3'	295
6	F: 5'CATGTCAATGGTTCCCCAGT 3' R: 5'TCCCACCATCTGCTTATTCA3'	361

Linkage association between loci was carried out using PLINK (Version 1.07) (Purcell et al. 2007).

The data on milk yield, milk fat, milk protein, milk lactose, and somatic cell count (SCC) in milk of the analysed cows were acquired based on monthly milk recording performed in compliance with the recommendations of the International Committee for Animal Recording (ICAR). Information on the age at first calving (AFC) and the episodes of clinical mastitis (CM) identified by the farm veterinarian was obtained from the farm documentation.

Depending on the incidence of CM, the cows were allocated into two groups (classes): resistant (class 1) and susceptible (class 2) to CM. Cows classified as resistant had no CM episodes in first three or four lactations. When CM occurred minimally three times in first three or four lactations, cow was classified as susceptible. To calculate frequencies of particular genotypes in each CM class, the FREQ procedure of SAS was applied.

Possible association between *SELL* gene polymorphism and the incidence of CM in first three or four lactations was examined using the LOGISTIC procedure of SAS by a logistic regression model defined as follows:

$$logit\ P = ln\ P/(1 - P) = b_0 + b_1G + b_2AFC + b_3L;$$
$$P = p(Y = 1)$$

where:

Y = occurrence of CM denoted as 1 – no episodes of CM and 2 – minimally three episodes of CM in first three or four lactations

b_0 = intercept

G = genotype (*AA*, *AG*, *GG* for c.165G>A or *CC*, *CT*, *TT* for c.567C>T) or haplotype combination (*AC/AC*, *GT/AC*, *GC/AC*, *GT/GC*)

AFC = age at first calving (≤ 24 months; > 24 to ≤ 26 months; > 26 months)

L = lactation number (1, 2, 3, 4, ≥ 5)

b_1–b_3 = regression coefficients of CM occurrence on fixed effects.

In order to interpret the logit model, odds ratios (OR), defined as odds of no episodes of CM to odds of minimum three episodes of CM in first three or four lactations, were used. Since class 1 (cows with no episodes of CM) was assumed as the reference, OR = 1 indicated equal odds of no episodes and minimally three episodes of CM in first three or four lactations, OR > 1 corresponded with the increased odds and OR < 1 with the decreased odds of no episodes of CM.

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The effect of *L-selectin* gene polymorphisms on SCC (log-transformed to somatic cell score, SCS = $\log_2$ (SCC/100 000) + 3) in milk of the studied cows was analyzed using the GLM procedure of SAS software with Scheffe's test, according to the linear model:

$$Y_{ijklmno} = \mu + G_i + AFC_j + L_k + LS_l + MYD_m + MY_n + e_{ijklmno}$$

where:

$Y_{ijklmno}$ = test-day somatic cell score
μ = overall mean
G_i = fixed effect of i^{th} genotype (*AA, AG, GG* for c.165G>A or *CC, CT, TT* for c.567C>T) or haplotype combination (*AC/AC, GT/AC, GC/AC, GT/GC*)
AFC_j = fixed effect of j^{th} age at first calving (≤ 24 months; > 24 to ≤ 26 months; > 26 months)
L_k = fixed effect of k^{th} lactation number (1, 2, 3, 4 ≥ 5)
LS_l = fixed effect of l^{th} lactation stage (1, 2, 3 ... ≥ 13 months)
MYD_m = fixed effect of m^{th} class of mean milk yield per milking day (≤ 25 kg; > 25 to ≤ 30 kg; >30 to ≤ 35 kg; > 35 kg)
MY_n = fixed effect of n^{th} class of test-day milk yield (≤ 25 kg; > 25 to ≤ 30 kg; > 30 to ≤ 35 kg; > 35 to ≤ 40 kg; > 40 kg)
$e_{ijklmno}$ = random error.

To investigate the effect of *SELL* polymorphism on milk traits, the GLM procedure of SAS software with Scheffe's test, using the following linear model, was applied:

$$Y_{ijklm} = \mu + G_i + AFC_j + L_k + LS_l + e_{ijklm}$$

where:

Y_{ijklm} = test-day milk yield (MY) or test-day milk fat percentage (MF) or test-day milk protein percentage (MP) or test-day milk lactose percentage (ML)
μ = overall mean of analyzed trait
G_i = fixed effect of i^{th} genotype (*AA, AG, GG* for c.165G>A and *CC, CT, TT* for c.567C>T or haplotype combination (*AC/AC, GT/AC, GC/AC, GT/GC*)
AFC_j = fixed effect of j^{th} age at first calving (≤ 24 months; > 24 to ≤ 26 months; > 26 months)
L_k = fixed effect of k^{th} lactation number (1, 2, 3, 4 ≥ 5)
LS_l = fixed effect of l^{th} lactation stage (1, 2, 3... ≥ 13 months)
e_{ijklm} = random error

RESULTS

Two SNPs within *L-selectin* gene – SNP c.165G>A in exon 3 (rs109966956 at 38, 170, 687 bp on chromosome 16) and a SNP c.567C>T in exon 4 (rs41803917 at 38, 168, 866 bp on chromosome 16) were detected in the studied population of the Holstein-Friesian breed. Both polymorphisms were synonymous variants. In the sequence of the remaining analysed exons, no SNPs were identified by the PCR-RFLP method. The c.165G>A PCR product digestion with *Bsm*I endonuclease resulted in uncut fragment (allele *G*) and the 99 and 400 bp restriction fragments (allele *A*). In the case of c.567C>T polymorphism, the PCR product digested with *Apa*I endonuclease revealed an uncut fragment (allele *T*) and cutting fragments of 91 and 190 bp (allele *C*).

In the studied herd, all possible *SELL* genotypes were found. The most frequent genotype for c.165G>A mutation was *AG* (0.51) and for c.567C>T mutation genotype *CC* (0.55). The most frequent alleles were *A* (0.59) and *C* (0.75), respectively. The frequencies of genotypes and alleles are presented in Table 2. The analysed population was in Hardy–Weinberg genetic equilibrium for both mutations (for SNP c.165G>A $P = 0.57$ and for SNP c.567C>T $P = 0.26$). The detected SNPs were not in linkage disequilibrium ($r^2 = 0.42$). Three *L-selectin* haplotypes (*AC, GT*, and *GC*) and six haplotype combinations (*AC/AC, GT/AC, GC/AC, GT/GT, GT/GC* and *GC/GC*) were identified in the examined cows. However, due to the lack of linkage disequilibrium between mutations, association analyses were not conducted for haplotype combinations.

Logistic regression analysis showed a statistically non-significant association between *L-selectin*

Table 2. Frequency of genotypes and alleles for mutations within L-selectin gene in the studied cows

Mutation	Genotype	*n*	Genotype frequency	Allele	frequency
c.165G>A	*AA*	68	0.33	*A*	0.59
	AG	104	0.51	*G*	0.41
	GG	33	0.16		
c.567C>T	*CC*	112	0.55	*C*	0.75
	CT	82	0.40	*T*	0.25
	TT	9	0.04		

n = number of animals

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Table 3. Least squares means (LSM) with standard errors (SE) for test-day somatic cell score (SCS), test-day milk yield (MY), test-day milk fat percentage (MF), test-day milk protein percentage (MP), and test-day milk lactose percentage (ML) in relation to different *L-selectin* genotypes

Mutation	Genotype	SCS (units)		MY (kg)		MF (%)		MP (%)		ML (%)	
		LSM ± SE	*P*-value	LSM ± SE	*P*-value	LSM ± SE	*P*-value	LSM ± SE	*P*-value	LSM ± SE	*P*-value
165G>A	*AA*	3.37 ± 0.06^{A}	0.0019	31.40 ± 0.14^{aB}	0.0001	3.80 ± 0.02^{AB}	< 0.0001	3.44 ± 0.006^{A}	< 0.0001	4.78 ± 0.004	0.0921
	AG	3.51 ± 0.06^{A}		30.91 ± 0.12^{a}		3.92 ± 0.01^{A}		3.48 ± 0.005^{A}		4.77 ± 0.004	
	GG	3.43 ± 0.07		30.43 ± 0.21^{B}		3.93 ± 0.02^{B}		3.52 ± 0.009^{A}		4.79 ± 0.006	
567C>T	*CC*	3.43 ± 0.05^{A}	0.0003	31.23 ± 0.12^{A}	< 0.0001	3.85 ± 0.01^{AB}	< 0.0001	3.45 ± 0.005^{A}	< 0.0001	4.70 ± 0.003^{A}	< 0.0001
	CT	3.42 ± 0.06^{B}		30.88 ± 0.14^{B}		3.92 ± 0.02^{A}		3.49 ± 0.006^{A}		4.80 ± 0.004^{A}	
	TT	3.05 ± 0.11^{AB}		27.07 ± 0.39^{AB}		4.01 ± 0.04^{B}		3.62 ± 0.016^{A}		4.78 ± 0.012	

$^{a–c}$means within each mutation with the same superscripts differ at $P \leq 0.05$

$^{A–C}$means within each mutation with the same superscripts differ at $P \leq 0.01$

genotypes and CM occurrence (for c.165G>A: Wald chi-square = 5.53; $P = 0.06$ and for c.567C>T: Wald chi-square = 2.50; $P = 0.29$).

According to the results obtained from the GLM models, both identified SNP polymorphisms affected SCS and milk production traits (Table 3). The effect of c.165G>A and c.567C>T mutations on SCS was highly significant ($P = 0.0019$ and $P = 0.0003$, respectively). In the case of c.165G>A mutation, the lowest SCS (Least squares means (LSM) = 3.37 ± 0.06) was in *AA* cows and this value differed highly significantly from that found in *AG* cows ($P \leq 0.01$). The differences in SCS between particular genotypes were observed also for c.567C>T polymorphism. SCS for *TT* cows (LSM = 3.05 ± 0.11) was highly significantly lower than values estimated for *CC* and *CT* cows ($P = 0.0003$ and $P = 0.0005$, respectively).

Both c.165G>A and c.567C>T mutation proved to have a highly significant ($P \leq 0.0001$) effect on test-day milk yield (MY), test-day milk fat percentage (MF), and test-day milk protein percentage (MP) while test-day milk lactose percentage (ML) was highly significantly ($P < 0.0001$) affected only by c.567C>T mutation. The effect of c.165G>A polymorphism on ML was not significant ($P = 0.0921$). Cows with *AA* genotype had the highest MY but the lowest MF and MP (in comparison with other c.165G>A genotypes). ML values were almost the same for each c.165G>A genotype. Similarly, in the case of c.567C>T polymorphism, cows with the highest MY (*CC* genotype) had the lowest MF and MP but, unlike c.165G>A mutation, also the lowest ML.

DISCUSSION

To date, the association between *SELL* gene polymorphism and mastitis in cows has not been investigated. However, some authors indicated the role of L-selectin in the inflammatory disease of mammary gland. Diez-Fraille et al. (2004) reported a progressive decrease of L-selectin expression on blood and milk neutrophils in response to infusion of *E. coli* lipopolysaccharides to the mammary gland. Similarly, research by Alhussien et al. (2016) showed that the relative mRNA expression of CD62L in blood neutrophils decreased significantly during mastitis ($P < 0.05$) compared to the level observed in healthy animals. In milk neutrophils this expression remained unaltered. The expression of CD62L

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during udder infection was also examined in Sahiwal cows in India. Healthy cows presented significantly higher L-selectin expression on blood and milk neutrophils than cows with clinical and subclinical mastitis (Swain et al. 2016). Contrarily, in the study by Karthikeyan et al. (2016), the expression level of L-selectin in milk somatic cells of cows affected by *S. aureus* subclinical mastitis was significantly higher ($P < 0.05$) than in healthy cows. This might indicate that the role of *SELL* gene in mastitis depends on the type of disease (*S. aureus* or *E. coli* infection).

All these findings about varied levels of L-selectin expression during mastitis in cows suggested that this molecule might play a major role in cows' susceptibility or resistance to this disease. However, in the present study the hypothesis about the influence of *SELL* gene polymorphism on CM occurrence in cows was not confirmed, possibly because of a relatively small number of animals covered by the analyses. Despite the logistic regression generally did not reveal a significant association between *SELL* polymorphism and CM, the effect of c.165G>A mutation was close to significance ($P = 0.06$). On the other hand, the results of GLM analysis demonstrated a highly significant effect of *L-selectin* genotypes on the level of SCS. Considering the well-known positive correlation between SCS and CM (Rupp and Boichard 1999), our results might indicate that the relationship between *L-selectin* gene polymorphism and udder health can be assumed reasonable and requires further investigation.

In the analysed set of cows, strong associations between the *L-selectin* SNPs and milk production traits were also observed. Assuming that *SELL* gene is involved in udder health in cattle, obtained results might be explained by the well-established genetic correlation between mastitis susceptibility and milk performance traits (Oltenacu and Broom 2010). Moreover, research by Edwards et al. (2015) showed that immune related pathways are associated with milk production traits in cattle. Alternatively, the mentioned associations may result from linkage between *L-selectin* polymorphism and polymorphisms in other genes or quantitative trait loci (QTL) on the same chromosome having a significant effect on milk production traits. *SELL* gene is located on BTA16. This chromosome harbours QTL for milk fat composition (Schennink et al. 2009) and milk yield (Iso-Touru et al. 2016), thus potential linkage disequilibrium between *L-selectin* SNPs and other genetic markers within BTA16 is possible.

CONCLUSION

The present research was the first to show the polymorphism within *L-selectin* gene in cattle. Although L-selectin appears to be biologically meaningful for mastitis in dairy cattle, the present study found no statistically significant effect of SNPs within this gene on CM occurrence in Polish Holstein-Friesian cows. However, due to strong associations observed between c.165G>A and c.567C>T polymorphisms and SCS, the role of *SELL* gene in udder health cannot be ruled out. Our study revealed also the relationship between the identified SNPs and economically important milk traits. This may be the result of linkage between these SNPs and other SNPs in *SELL* or other genes located on the same chromosome region significantly affecting the mentioned traits.

All associations identified in this study require further investigation before they can be considered as genetic markers in cattle selection.

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