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Identification of polymorphism in the bovine interleukin-17A gene and its association with mastitis in Polish Holstein–Friesian cattle

Magdalena DUSZA*, **Joanna POKORSKA**, **Dominika KUŁAJ**, **Joanna MAKULSKA**
Department of Cattle Breeding, University of Agriculture in Krakow, Krakow, Poland

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Abstract: The main aim of this study was to identify the association between the single nucleotide polymorphism (SNP) in the bovine interleukin-17A (IL-17A) gene and mastitis resistance. Additionally, the connection between the identified polymorphism and the somatic cell score (SCS) was tested. Two hundred and six Polish Holstein–Friesian cows were genotyped using the PCR-RFLP method. One SNP mutation (c.126G>A) was identified within the coding sequence of the analyzed gene. Results of logistic regression analysis displayed no significant association between the IL-17A genotype and clinical mastitis occurrence (Wald chi-square test = 0.74; P = 0.69). The GLM analysis demonstrated that genotype also did not affect the SCS (P = 0.77), while the effects of age at first calving, lactation number, lactation stage, mean milk yield per milking day, and milk yield on the day of milk recording proved to be highly significant (P < 0.01). The present study was one of the first to show an association between polymorphism within the IL-17A gene and selected udder health traits in cattle. An influence of the c.126G>A polymorphism on clinical mastitis and the SCS in the studied set of Polish Holstein–Friesian cows was not found. This suggests that the c.126G>A marker is probably not relevant for genetic selection against mastitis in the analyzed breed.

Key words: Interleukin-17, mastitis, somatic cell count, cattle

1. Introduction

Intensive genetic selection in dairy cattle has resulted in a great increase in milk performance. In many countries, milk production per cow has more than doubled in the last 40 years (1). This has not been without effects on the welfare and health of the animals. The review by Ingvartsen et al. (2) revealed an unfavorable genetic correlation between milk yield and incidence of common bovine diseases like ketosis (0.26–0.65), ovarian cysts (0.23–0.42), mastitis (0.15–0.68), and lameness (0.24–0.48). Furthermore, the fertility of dairy cows has declined considerably in the last decades (3). Deteriorated reproductive performance and higher frequency of diseases at the beginning of lactation in high-milking cows may be connected to a negative energy balance resulting from insufficient dietary energy intake (4).

Mastitis results in an immunological response characterized by rapid increase of the somatic cell count (mainly white blood cells: neutrophils, macrophages, and lymphocytes) in the milk (5). This response of the immune system depends on the action of inflammatory mediators like cytokines, chemokines, prostaglandins, and leukotrienes (6). Cytokines are key signaling molecules for communication in the immune system. The interleukin-17 (IL-17) family is a recently identified group of cytokines involved in the maintenance of homeostasis and host defense against bacterial infections. The prototype cytokine of IL-17 family is interleukin-17A, produced mainly by activated CD4+ T cells (but also by T cells, natural killer cells, lymphoid tissue inducer-like cells, and neutrophils); it plays a significant role in promoting inflammation by recruitment of neutrophils during infection (7). Reinard et al. (8) revealed that the production of IL-17A by CD4+ T cells is a predictor of the antigen-specific immune response of the cow mammary gland. The importance of IL-17 for mastitis occurrence was also demonstrated in the findings of Jing et al. (9), who observed the elevated level of this cytokine in the milk of goats experimentally infected with *Escherichia coli* or *Staphylococcus aureus*.

However, research on interleukin-17A is still very scarce. In the present study, the genetic polymorphism within a bovine IL-17A gene was analyzed. The impact of the identified single nucleotide polymorphism (SNP) on the resistance or susceptibility to clinical mastitis and on the level of the somatic cell score (SCS) in Polish Holstein–Friesian cows was then examined.

* Correspondence: duszamagda@gmail.com

2. Materials and methods

The study was performed on 206 cows of the Polish Holstein–Friesian breed, Black and White strain, being the daughters of 97 sires. The cows were maintained at one farm in identical environmental conditions and were fed a total mixed ration. All cows had completed at least the first three lactations.

Genomic DNA for molecular analyses was isolated from the cows' milk using an original method developed at the Department of Cattle Breeding, Faculty of Animal Sciences, University of Agriculture in Krakow, as described by Pokorska et al. in 2015 (10). The qualitative and quantitative analysis of the isolated DNA was carried out using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Genotypes of the cows were determined with the PCR-RFLP method. Primers for PCR (Table 1) were designed based on the reference sequence (GenBank: NM_001008412.2). Preliminary analysis of polymorphism included all three exons of the bovine IL-17A gene.

PCR was performed in a final volume of 20 µL, containing approximately 150 ng of template DNA, 2 µL of 10X 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 of Taq polymerase. All reagents were supplied by Thermo Scientific. PCR was carried out on a C1000 Thermal Cycler (Bio-Rad).

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 62 °C for 40 s, and elongation at 72 °C for 50 s). The final elongation was carried out at 72 °C for 7 min.

The amplified products for 5 sample cows were sequenced by a commercial company (Genomed S.A., Warsaw, Poland) to perform preevaluation of potential SNPs existing in the analyzed sequence of the IL-17A gene. Sequences for sample cows were compared with the reference sequence and with each other to identify SNP. For the preliminary identified polymorphism, a restriction enzyme was selected.

The amplified products for all cows were digested with restriction enzyme *Aci*I (Thermo Scientific) as recommended by the manufacturer. The obtained restriction fragments were separated in 3% agarose gel stained with SYBR Safe dye (Invitrogen).

Genotypic and allelic frequencies were calculated using the FREQ procedure of SAS (SAS Institute Inc., v. 9.2). The Hardy–Weinberg equilibrium of the mutation was determined by chi-square test.

The data on milk yield (kg) and somatic cell count in milk (SCC; thousands/mL) of the analyzed cows were acquired based on monthly milk recordings performed in compliance with the recommendations of the International Committee for Animal Recording. Information on age at first calving (AFC) and episodes of clinical mastitis (CM), as identified by the farm veterinarian, was obtained from the farm's documentation. The data on CM were available for only 198 cows from among the 206 genotyped cows.

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

Until the end of the second lactation, all CM episodes were recorded. In cows having fewer than three episodes of CM in the first two lactations, recording was continued maximally to the end of the fourth lactation. However, as soon as three episodes of CM were observed, a cow was considered susceptible and recording was no longer carried out.

In order to evaluate the difference between the number of CM episodes in the first two lactations in cows with different IL-17A genotypes, preliminary analysis was performed using the nonparametric Kruskal–Wallis test with the NPAR1WAY procedure of SAS software. Although the obtained results did not indicate a significant effect for genotype, the final verification of a possible association between IL-17A gene polymorphism and the incidence of CM was carried out using the data from the first three or four lactations. The PROC LOGISTIC of SAS, by a logistic regression model defined as follows, was applied:

$$\text{logit } P = \ln\frac{P}{1-P} = b_0 + b_1 G + b_2 AFC + b_3 L$$

where P = p (Y = 1); Y is the occurrence of CM denoted as 1 - no episodes of CM and 2 - minimum of 3 episodes of CM in first 3 or 4 lactations; b_0 is the intercept, G is genotype

Table 1. Selected PCR conditions for exon 2 of the bovine IL-17A gene.

Exon number	Primer sequence	Annealing temp.	Product size (bp)
Exon 2	F: 5′ TGTCCTGGACCATAGAATGTTCT 3′ R: 5′ TGGCTCTTCCAGGTTTGACA 3′	62 °C	434

(AA, AG, GG); AFC is age at first calving (≤24 months; >24 to ≤26 months; >26 months); L is lactation number (1, 2, 3, 4, ≥5); and b_1–b_3 are regression coefficients of CM occurrence on fixed effects.

In order to interpret the logit model, the odds ratio (OR), defined as odds of no episodes of CM to odds of a minimum of 3 episodes of CM in the first 3 or 4 lactations, was used. Since class 1 (cows with no episodes of CM) was assumed as the reference, OR = 1 indicated equal odds of no episodes and a minimum of 3 episodes of CM in the first 3 or 4 lactations, OR >1 corresponded with increased odds and OR <1 with decreased odds of no episodes of CM.

The effect of interleukin-17A gene polymorphisms on the SCC (log-transformed to somatic cell score, $SCS = \log_2 [SCC/100{,}000] + 3$) in the milk of the studied cows was analyzed using PROC GLM of SAS software with Scheffe's test, according to the following linear model:

$$Y_{ijklmno} = \mu + G_i + AFC_j + L_k + LS_l + MYMD_m + MYRD_n + e_{ijklmno},$$

where $Y_{ijklmno}$ is SCS on the day of milk recording; μ is the overall mean; G_i is a fixed effect of the IL-17A *i*th genotype (AA, AG, GG); AFC_j is a fixed effect of *j*th age at first calving (≤24 months; >24 to ≤26 months; >26 months); L_k is a fixed effect of *k*th lactation number (1, 2, 3, 4, ≥5); LS_l is a fixed effect of the *l*th lactation stage (1, 2, 3... ≥13 months); $MYMD_m$ is a fixed effect of the *m*th class of mean milk yield per milking day (≤25 kg; >25 to ≤30 kg; >30 to ≤35 kg; >35 kg); $MYRD_n$ is a fixed effect of *n*th class of milk yield on the day of milk recording (≤25 kg; >25 to ≤30 kg; >30 to ≤35 kg; >35 to ≤40 kg; >40 kg); and $e_{ijklmno}$ is a random error.

3. Results

In 206 studied cows, all three exons of the interleukin-17A gene were analyzed to find genetic polymorphisms, but only one SNP was identified within the gene coding sequence. On the basis of the received sequencing chromatograms (Figure 1), a polymorphic site was identified and the appropriate restriction enzyme (*Aci*I with recognition site 5′-C↓CGC-3′) was selected to conduct further genotype analysis in all cows. The PCR product (434 bp) digestion with *Aci*I endonuclease resulted in a noncutting fragment (allele A) or the 205-bp and 229-bp restriction fragments (allele G) (Figure 2).

The SNP c.126G>A was localized in the second exon at 24,348,328 bp on chromosome 23 and was a synonymous variant (codon change from CCA to CCG, both coding the amino acid proline). The official NCBI name of this mutation is rs209908719 (according to the dbSNP database: www.ncbi.nlm.nih.gov/SNP/).

The most frequent allele for the c.126G>A mutation was A (0.63), and the most frequent genotype was AG (0.50). The frequencies of genotypes and alleles are presented in

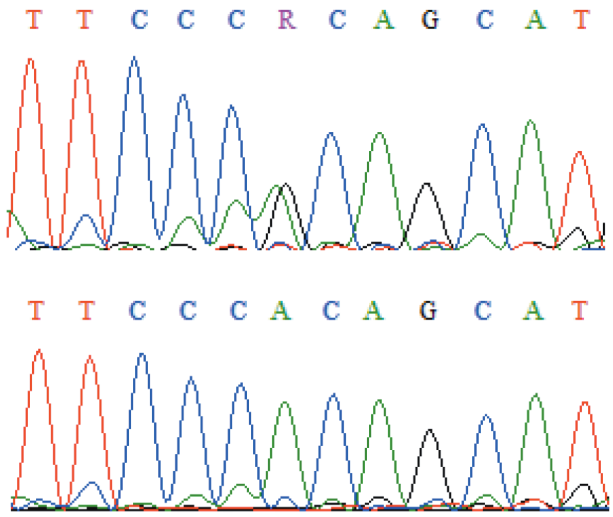

Figure 1. The sequencing chromatograms of the bovine IL-17A gene indicating a polymorphic site in exon 2 (at c.126A>G) (upper chromatogram: letter R meaning A or G allele; lower chromatogram: A allele at the same site).

Table 2. The studied population was in Hardy–Weinberg genetic equilibrium (P = 0.45). The mean numbers of CM episodes in the first 2 lactations in each genotype group are presented in Table 3. The numbers of cows resistant and susceptible to mastitis were almost equal (Table 4). Similarly, particular IL-17A genotypes occurred with nearly the same frequency in both CM classes.

The results of the Kruskal–Wallis test did not show statistical differences between the number of CM episodes in the first two lactations of the cows with AA, AG, and GG genotypes (P = 0.85).

Logistic regression analysis performed on the data including the episodes of CM until the third or fourth lactation proved the statistical insignificance of the association between IL-17A genotypes and CM occurrence (Wald chi-square test = 0.74; P = 0.69).

Polymorphism in the IL-17A gene also did not affect SCS in the investigated cows (P = 0.77), while the effects of age at first calving, lactation number, lactation stage, mean milk yield per milking day, and milk yield on the day of milk recording were highly significant (P < 0.01) (Table 5).

The cows in the youngest AFC class (age at first calving ≤24 months) had the highest value of SCS, while the cows in the oldest AFC class (age at first calving >26 months) had the lowest SCS. Somatic cell score increased in subsequent lactations, with the highest value in the fifth lactation class, including the fifth and later lactations (LSM = 4.05 ± 0.08, compared to 2.63 ± 0.07 in the first lactation). The lowest SCS values were observed in the second and third months of lactation (LSM = 2.95 ± 0.08 and 3.06 ± 0.08, respectively). They then constantly increased, reaching

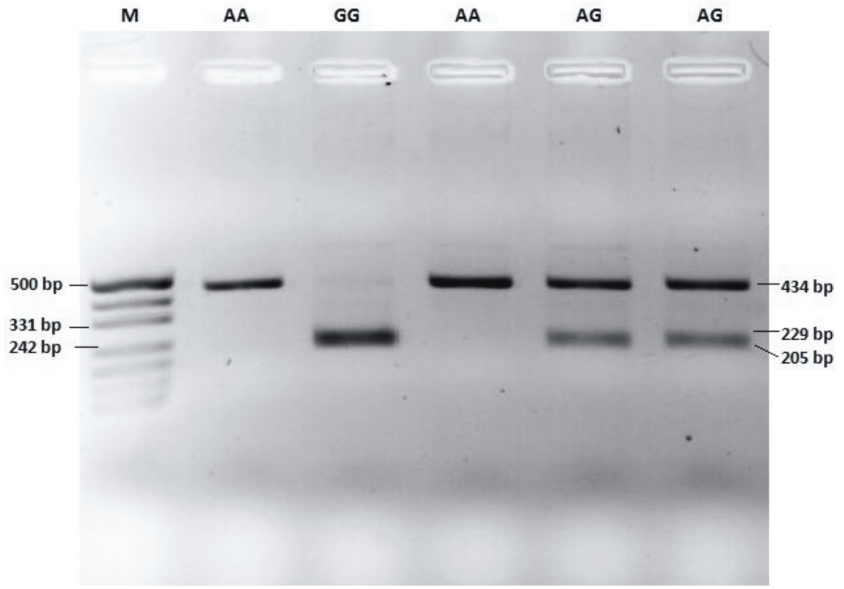

Figure 2. PCR-RFLP detection of the c.126A>G polymorphism in the bovine IL-17A gene; M – pUC19 DNA Ladder (Thermo Scientific); AA, GG, AG – individual c.126A>G genotypes.

Table 2. Frequency of genotypes and alleles for c.126G>A mutation in the studied cows.

Genotype	N	Genotype frequency	Allele frequency	
AA	78	0.38	A	0.63
AG	102	0.50	G	0.37
GG	26	0.12		

N - number of animals.

Table 3. Mean number of clinical mastitis (CM) episodes in first two lactations of the cows with different IL-17A genotypes.

IL-17A genotype	N	Mean number of CM episodes
AA	74	1.28
AG	98	1.12
GG	26	1.19
All	198	1.20

N - number of animals.

Table 4. Distribution of AA, AG, and GG genotypes in clinical mastitis (CM) classes.

	IL-17A genotype			
CM class*	AA	AG	GG	All
1	40	48	12	100
2	34	50	14	98
All	74	98	26	198

*CM class: 1 – cows resistant to mastitis, 2 – cows susceptible to mastitis.

the highest value of 3.95 ± 0.15 in the thirteenth stage, including the thirteenth and later months. SCS was also associated with the level of milk production. High-yielding cows (with mean milk yield per milking day of >35 kg) had the highest value of SCS, but the highest milk yield on the day of milk recording was at the lowest value of SCS.

4. Discussion

Studies on the genetic polymorphism of the interleukin-17A gene in Polish Holstein–Friesian cattle have revealed only one SNP mutation in the coding sequence of this gene: c.126G>A in the second exon. According to the NextGen Project (http://projects.ensembl.org/nextgen/),

Table 5. Least squares mean (LSM) with standard error (SE) and 95% confidence interval (CI) for somatic cell scores.

Effect	Class	N	LSM ± SE	95% CI		P-value
				Lower	Upper	
SNP	AA	3195	3.43 ± 0.06	3.32	3.54	0.7672
	AG	4366	3.43 ± 0.06	3.32	3.54	
	GG	1128	3.47 ± 0.07	3.33	3.61	
Age at first calving (months)	≤24	2279	3.53 ± 0.06^{ab}	3.41	3.65	0.0099
	>24 to ≤26	4746	3.41 ± 0.05^{a}	3.30	3.51	
	>26	1664	3.39 ± 0.07^{b}	3.26	3.52	
Mean milk yield per milking day (kg)	≤25	83	3.12 ± 0.19^{a}	2.74	3.49	<.0001
	>25 to ≤30	2136	3.39 ± 0.05^{AB}	3.31	3.48	
	>30 to ≤35	4072	3.58 ± 0.03^{A}	3.52	3.65	
	>35	2398	3.67 ± 0.04^{Ba}	3.60	3.75	
Milk yield on the day of milk recording (kg)	≤25	1878	4.11 ± 0.06^{AB}	3.99	4.24	
	>25 to ≤30	1584	3.65 ± 0.07^{AB}	3.53	3.79	
	>30 to ≤35	1656	3.42 ± 0.07^{AB}	3.29	3.55	
	>35 to ≤40	1312	3.13 ± 0.07^{aA}	2.98	3.27	
	>40	2259	2.90 ± 0.08^{aB}	2.76	3.05	
Lactation number	1	2063	2.63 ± 0.07^{ABCD}	2.50	2.77	<.0001
	2	2102	3.12 ± 0.06^{ABCD}	2.99	3.24	
	3	2102	3.55 ± 0.06^{ABCD}	3.43	3.67	
	4	1550	3.86 ± 0.07^{ABC}	3.73	3.99	
	≥5	872	4.05 ± 0.08^{ABC}	3.90	4.20	
Lactation stage (month)	1	716	3.30 ± 0.08	3.14	3.46	
	2	826	2.95 ± 0.08^{AE}	2.79	3.12	
	3	825	3.06 ± 0.08^{BF}	2.90	3.21	
	4	813	3.20 ± 0.08^{CG}	3.04	3.35	
	5	808	3.27 ± 0.08^{DHa}	3.12	3.43	
	6	807	3.42 ± 0.08^{ABCDIJKM}	3.27	3.57	
	7	802	3.46 ± 0.08^{EFGHbLc}	3.31	3.62	
	8	796	3.53 ± 0.08^{a}	3.38	3.69	
	9	774	3.52 ± 0.08	3.36	3.66	
	10	641	3.63 ± 0.09^{I}	3.46	3.81	
	11	433	3.71 ± 0.10^{Jb}	3.50	3.91	
	12	278	3.76 ± 0.12^{KL}	3.52	4.00	
	≥13	170	3.95 ± 0.15^{Mc}	3.66	4.24	

N - number of observations in each class.
$^{a,b,c...}$ Means within each effect with the same superscripts differ at P ≤ 0.05.
$^{A,B,C...}$ Means within each effect with the same superscripts differ at P ≤ 0.01.

mutation c.126G>A has been previously detected in the Iranian cattle population. The frequency of the G allele in Iranian cows was 0.75, and the frequency of the A allele was 0.25. These results were opposite to those obtained in the present study, where the A allele was more frequent than the G allele.

To date, studies indicate that there are a few common SNPs in the human interleukin-17A gene associated with various diseases, e.g., cardiovascular (coronary artery disease) (11) and gastrointestinal diseases (12). Furthermore, some research has confirmed the association between the rs1974226 SNP in the IL-17A gene and altered

susceptibility to gram-positive infections in patients with severe sepsis (13). It has been proved that abnormal expression of IL-17A is related to autoimmune diseases and cancer (14). Ishigame et al. (15) observed an increased susceptibility to *Staphylococcus aureus* skin infection in transgenic mice lacking IL-17A. Blanco et al. (16) revealed that elevated expression of IL-17 is connected with tuberculosis.

The contribution of interleukin-17A to the inflammation of cow mammary glands has been investigated in a few studies. Transcripts of IL-17A were found in somatic cells from *Staphylococcus aureus*-infected cows (17). Analyses of the transcriptomic profiles of the initial bovine mammary epithelial cells' response when stimulated with *S. aureus* culture showed that these bacteria induce the IL-17A proinflammatory pathway (18). Moreover, the expression of the bovine receptor for interleukin 17A (IL-17RA), which is associated with the functioning of this cytokine pathway, was detected in healthy mammary epithelial cells (19). Tassi et al. (20) demonstrated production of IL-17A in cows with *S. uberis* mastitis. Recent research by Roussel et al. (21) showed overexpression of the IL-17A gene in the udder during *E. coli* mastitis. These authors demonstrated a potential role for interleukin-17A in enhancing antimicrobial response of mammary epithelial cells after *E. coli* infection. Additionally, the findings of Usman et al. (22) indicated that IL-17A may be one of the agents contributing to immune response and mastitis resistance in cattle. However, this hypothesis was not confirmed in the present study, possibly because of the relatively small number of animals covered by the analyses.

In our investigations, statistically significant differences in the SCS depending on age at first calving were observed. The highest values of SCS in the youngest AFC class may be connected with the fact that, according to some authors (23), cows calved earlier (but not before 22 months of age) tend to have higher milk production, and, as has been shown before, higher milk production is associated with elevated SCS. This may also be due to possibly decreased immune functions in cows with reduced AFC.

It has been found that the level of SCS was significantly affected by the lactation number. Cows in fifth and higher lactations had SCS levels almost twice as high as primiparous cows. These findings are consistent with previous observations (24,25).

SCS also changed significantly over the course of lactation. It was quite high in the first month, then dropped in the second month, increasing gradually afterwards until the end of lactation. An elevated SCS at the beginning of lactation can be associated with the negative energy balance often affecting high-milking cows. In order to meet the increased energy demand for milk production, the cows need to mobilize body adipose reserves (26), which can increase their susceptibility to diseases like mastitis. Similar to the present study, Koç (27) reported a high SCC in the first month of lactation, which decreased in the second month and then increased towards the last month of lactation. This could be explained by a dilution effect, which means that a specified number of somatic cells is diluted in a higher volume of milk produced at the peak of lactation (28,29). In addition, the dilution effect can explain the decrease of SCS with increasing milk yield on the day of milk recording, as revealed in this study. On the contrary, the high-milking cows (mean milk yield per milking day of >35 kg) tended to have an increased SCS compared with the cows characterized by lower mean milk yield per milking day. Since the increased level of SCS may be an indicator of udder inflammation (30), high-yielding cows may be less resistant to mastitis, which, in fact, has been observed by many cattle breeders (31).

In conclusion, the obtained results showed no significant effect of c.126G>A polymorphism on either clinical mastitis or SCS in the milk of Polish Holstein–Friesian cows. Thus, it can be suggested that this marker is not relevant for genetic selection against mastitis in the mentioned breed. The performed analyses revealed that reduced age at first calving was associated with higher SCS, which indicates that overly early first calving may not be advantageous for udder health. However, further studies are necessary to confirm this hypothesis.

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