Ann. Anim. Sci., Vol. 22, No. 2 (2022) 575–581 *DOI: 10.2478/aoas-2021-0061*

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EPIGENETIC STATES OF GENES CONTROLLING IMMUNE RESPONSIVENESS IN BOVINE CHRONIC MASTITIS*

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Abstract

Mastitis is a common disease in dairy cows, causing substantial economic losses. The leading cause of elevated milk somatic cell count (SCC), which is the best indicator for detecting mastitis, is the invasion of pathogens. A major pathogen responsible for bovine mastitis is *Staphylococcus aureus*, a member of the coagulase-positive staphylococci. Some strains of coagulase-negative staphylococci can also be a cause of clinical or subclinical mastitis. Our study used bisulfite sequencing PCR (BSP) to detect the methylation status of nine candidate genes (*CCL2*, *HCK*, *F11R*, *CD8A*, *PDIA3*, *LGMN*, *HSPA1A*, *IL18* and *NFKBIA*). We investigated the mechanisms associated with overexpression of these genes, in the mammary gland secretory tissue of cows diagnosed with mastitis and infected with coagulase-positive or coagulase-negative staphylococci. The results showed no changes at the DNA methylation level between the mastitis (CoPS and CoNS) and control groups (H), except for in the *HCK* region, where the observed differences between the CoPS and H groups were statistically significant. The low methylation level of the CpG sequence seems not to correspond to the previously observed increased activity of these genes, suggesting that mechanisms other than DNA methylation may control mRNA expression at the analyzed loci.

Key words: BSP, cattle, DNA methylation, mastitis

Inflammation of the mammary gland tissue of dairy cows has become a major concern to the dairy industry, as it adversely affects milk quantity, quality and composition, and also endangers public health (Bradley, 2002; Hagnestam-Nielsen et al., 2009; Viguier et al., 2009). This condition disrupts the physiological functioning of the mammary gland (Bostedt, 2001), resulting in increased milk somatic cell count (SCC) (Thompson-Crispi et al., 2014). The best indicator for detecting bovine mastitis is SCC in milk, which reflects the health status of the udder and the hygienic quality of milk (Leitner et al., 2000). It is now generally accepted that mastitis is a multifactorial disease, with the main causative agent being microorganisms. Of around 150 species of microorganisms that have been identified (Watts, 1988), the main etiological agents include *Staphylococcus aureus* (coagulase-positive bacteria), coagulase-negative staphylococci, *Streptococcus agalactiae*, environmental streptococci, *Escherichia coli* and other coliforms, and *Corynebacterium bovis* (Malinowski et al., 2006; Fijałkowski et al., 2008). Bovine mastitis is considered to be the most costly infectious disease in the dairy industry (Rainard et al., 2016). Many programs have been developed to improve immunity and reduce the incidence of mastitis in cattle. However, conventional strategies based on phenotypic selection are still rather inefficient, due to the low heritability of mastitis susceptibility (Hinchs et al., 2011). Thus, it is the task of scientists to find a tool to improve cow udder health, while maintaining high milk production. To this end, many studies are conducted worldwide, that include genomic, transcriptomic and epigenetic analyses (Ogorevc et al., 2009; Wang et al., 2013; Chang et al., 2015; Song et al., 2016; Kościuczuk et al., 2017; Zhang et al., 2018; Wang et al., 2019; Ząbek et al., 2020). The use of expression microarrays has revealed aberrant expression of many genes involved in immune response mechanisms associated with bovine mastitis (Kościuczuk et al., 2017). DNA methylation is one of the main mechanisms of epigenetic modification that plays a key role in the expression of genes. Previous studies have proven that epigenetic changes are related to mastitis (Wang et al., 2013; Chang et al., 2015; Song et al., 2016; Zhang et al., 2018; Wang et al., 2019). Therefore, the aim of this study was to describe the DNA

*The study was supported by the grant of the Polish Academy of Sciences No. 2015/17/B/NZ9/01561.

methylation status of the genes of the chemokine signaling pathway (C-C motif chemokine 2 – *CCL2,* tyrosine-protein kinase HCK – *HCK*), the cell adhesion molecules pathway (junctional adhesion molecule A – *F11R*), the antigen processing and presentation pathway (T-cell surface glycoprotein CD8 alpha chain – *CD8A,* protein disulfide-isomerase A3 – *PDIA3,* Legumain – *LGMN,* heat shock 70 kDa protein 1A – *HSPA1A*) and the NOD-like receptor signaling pathway (interleukin-18-*IL18,* NF-kappa-B inhibitor alpha – *NFKBIA*), the differential diagnosis of which has been proven significant for mammary gland infection in cattle (Kościuczuk et al., 2017).

Material and methods

Animals and sample collection

The study was performed with Polish Holstein-Friesian dairy cows of the Black-and-White variety, which were slaughtered in a certified abattoir under constant veterinary supervision. Animals were culled on approximately the 280th day of lactation (SD=25) due to chronic mastitis (several unsuccessful antibiotic therapies in the last lactation were conducted), or due to reproduction problems without udder infection. Animals with clinical forms of mastitis were excluded from the study. Samples of mammary gland parenchyma tissue, with a predominance of secretory tissue, were collected from each udder quarter immediately after slaughter. The mammary gland secretory tissue health status was determined, based on microbiological analysis of "foremilk" samples collected aseptically two days before the slaughter.

DNA methylation levels were analyzed using 50 biological quarter samples, either infected with coagulase-positive staphylococci (CoPS; N = 26), coagulase-negative staphylococci (CoNS; N = 13), or not infected with any bacteria (H; N = 11). The samples assigned to the control group (H) were from cows in which the whole udder was healthy, to avoid cross-infection of adjacent quarters. Detailed information about the animals and their maintenance, and the sampling and microbiological analysis of tissues was previously reported by Kościuczuk et al. (2014).

Genomic DNA was isolated from the tissues using a commercial kit (Macherey Nagel, Düren, Germany). The purified DNA was then quantified and quality assessed by NanoDrop 2000 spectrophotometer, then was subjected to bisulfite conversion with the EpiTect Bisulfite Kit (Qiagen). All of the procedures followed the manufacturers' protocols.

Bisulfite sequencing

We selected a panel of nine candidate genes (*CCL2, HCK, F11R, CD8A, PDIA3, LGMN, HSPA1A, IL18, NFKBIA*), which in earlier analyses using expression microarrays (Bovine (V2) Gene Expression Microarrays 4 × 44K, Agilent, USA) revealed differences in expression level in non-infected mammary gland secretory tissue vs. those infected with coagulase-positive or coagulase-negative staphylococci (Kościuczuk et al., 2017). The potential gene promoter regions were selected using MatInspector software (Genomatix). The methylation level of CpG sites in selected genes was determined by bisulfite sequencing PCR (BSP), with primers designed with Methyl Primer Express® Software v1.0 (Applied Biosystems Software). The amplification reaction was performed in 20 µl volumes, using Hot Start Taq® polymerase (Qiagen). A two-round BSP amplification protocol was used. The thermal amplification conditions and the primer sequences are presented in Table 1.

Table 1. Primer sequences used in BSP reaction

Gene	Number of analyzed CpG sites	Primer sequences (5'–3')	Annealing temp [°C] (5 and 35 cycles)
CCL2 (Gene ID: 100034136)	7	F: GGGTGGATTAGTTATTTGTTTA R: ACCTAACTAAACRAACACCTC	58/56
HCK (Gene ID: 100068557)	22	F: TTTGGGGTAGAATTAAGATTTT R: AACCCTCTAACRCTAAACTATC	58/56
F11R (Gene ID: 100066720)	26	F: GTGGAGTGGGTAGAGGAATATA R: CCCTAAAAACAAACACCAAC	58/56
CD8A (Gene ID: 100066774)	20	F: GGTGTTAGGGTTATTTTGAATG R: ACTCACCRAACAACAACAA	58/56
PDIA3 (Gene ID: 100056198)	46	F: TAGAGGGAGGGTTTAAGAATTG R: ACCTACAACCRAAAATCCC	58/56
LGMN (Gene ID: 100053265)	26	F: TTTTTAGGGAGAGGTAGAGGT R: TCCTCCCTACAAAAATTACTAAA	56/54
HSPA1A (Gene ID: 100050827)	17	F: TTTTGAGTTGGGTTTAGATTTAG R: AATTTTATCAACTCTCTCCAAACT	58/56
IL18 (Gene ID: 100034216)	8	F: AAGAGGTATAAATTTTGAAGGGT R: ATTTAAAATCCCTTTCTTTTCC	56/54
NFKBIA (Gene ID: 100058000)	15	F: GAAATGATTTGAGGGTTTTTTA R: ACACACAATAATCCTAACCCTC	58/56

Table 2. The mean percentage of DNA methylation within analyzed gene regions, determined using the Mquant method

Group		Mean methylation percentage in the studied groups								
		CCL2	*HCK*	*F11R*	*CD8A*	*PDIA3*	*LGMN*	*HSPA1A*	*IL-18*	*NFKBIA*
H*		16.71	26.65	15.61	13.44	11.83	20.20	11.77	8.96	8.32
CoPS*		18.15	26.16	15.08	14.75	11.75	20.35	10.28	8.80	7.93
CoNS*		19.12	24.53	16.15	15.03	11.20	20.15	11.31	9.11	7.65
Difference CoPS vs H		1.44	0.49	0.53	1.31	0.08	0.15	1.49	0.16	0.54
Difference CoNS vs H		2.41	2.12	0.54	1.59	0.63	0.05	0.46	0.15	0.67
Shapiro-Wilk test (P-value)	H	1.74e-08	4.87e-11	0.001	1.64e-05	2.13e-11	7.41e-15	1.39e-07	2.51e-06	1.46e-15
	CoPS	3.36e-15	< 2.2e-16	4.13e-09	< 2.2e-16	< 2.2e-16	< 2.2e-16	1.92e-15	5.36e-11	< 2.2e-16
	CoNS	2.70e-10	1.68e-11	8.10e-05	7.20e-10	2.13e-11	< 2.2e-16	1.52e-10	1.30e-07	2.14e-14
Mann–Whitney U test (P-value)	CoPS vs H	0.45	0.05	0.36	0.29	0.91	0.91	0.27	0.70	0.76
	CoNS vs H	0.42	0.41	0.59	0.11	0.46	0.73	0.88	0.93	0.76
	CoPS vs CoNS	0.91	0.25	0.09	0.48	0.43	0.80	0.25	0.66	0.86

*CoPS – udder quarters infected with coagulase-positive staphylococci; CoNS – udder quarters infected with coagulase-negative staphylococci; H – udder quarters not infected with any bacteria (control group)

After amplification, BSP products were exposed to exonuclease I and alkaline phosphatase (Thermo Fisher Scientific), and sequenced using a BigDye® Terminator v3.1 kit (Thermo Fisher Scientific), according to the standard protocol. The sequencing products were electrophoresed on a 24-capillary 3130xl genetic analyzer (Applied Biosystems).

Analysis of BSP results

Chromatograms were analyzed using FinchTV v1.4.0 (Geospiza Inc.). This program was also used to reject low-quality reads, and for qualitative analysis of DNA methylation, thus confirming the presence of cytosine or thymidine at the analyzed CpG sites. The methylation status of CpG sites was estimated using the Mquant method described by Leakey et al. (2008), based on fluorescence intensity values of the different peaks shown in the sequencing chromatogram (values generated by BioEdit software) (Hall, 1999). Statistical differences in DNA methylation level were determined using the Shapiro-Wilk normality test and Mann–Whitney U test (R Development Core Team, 2011).

Results

The analysis of 187 CpG sites did not show significant differences in methylation level between the analyzed groups for any of the examined gene fragments (P>0.05), except for in the *HCK* region, where the observed differences between CoPS and H groups were statistically significant (P=0.05) (Table 2). The results revealed a low level of DNA methylation, ranging from 7.04 to 9.00% per sample (mean 7.65% ± 9.94 SD) for the *NFKBIA* region within the CoNS group, to 23.03–30.28% (mean 26.16% ± 16.66 SD) for the *HCK* region within the CoPS group. The Mquant method showed the same DNA methylation trend for the analyzed samples infected by coagulase-positive or coagulase-negative staphylococci, and also for the control group. There was a similar DNA methylation percentage across groups (differences between groups ranged from 0.05% to 2.41% and were statistically insignificant). Table 2 presents detailed results of the methylation analysis for all gene fragments.

Discussion

Bovine mastitis caused by *S. aureus* constitutes a major problem for the dairy industry and public health. Previous research has demonstrated that pathogens may contribute to epigenetic changes in the host, giving rise to certain diseases (Al Akeel, 2013). Therefore, we used the BSP method to study possible epigenetic changes in the selected panel of genes in the mammary gland secretory tissue of cows diagnosed with mastitis. In another prior study, Kościuczuk et al. (2017) reported overexpression of the *CCL2, IL18, HCK, F11R, CD8A, PDIA3, LGMN, HSPA1A* and *NFKBIA* loci in cows affected with mastitis, which may suggest that these genes play a crucial role in the development of this disease. It is now clear that long-lasting inflammation may be detrimental to the host and promote a permanent disease state, resulting in gene expression dysregulation and tissue damage (Huang et al., 2014). Research studies have so far indicated that bovine mastitis leads to the strong release of pro-inflammatory cytokines, which play key roles in the regulation of defense responses, including increasing macrophage and neutrophil bactericidal capacity, promoting the recruitment of neutrophils towards the site of infection, inducing the maturation of dendritic cells, and controlling the acquired immune response (Oviedo-Boyso et al., 2007). However, most prior studies have been conducted on the acute phase of the inflammation model. The protein products of the genes investigated in our study play a pivotal role in the immune response. For example, they take part in the recruitment of monocytes, memory T-cells and dendritic cells to the sites of inflammation (Carr et al., 1994; Xu et al., 1996), enhance the secretion of growth factors and pro-inflammatory cytokines (Poh et al., 2015), or are involved in T-cell activation (Miceli and Parnes, 1991), and in the processing of proteins for MHC class II antigen presentation in the lysosomal system (Dall and Brandstetter, 2016).

The results obtained here showed a low level of DNA methylation in predicted regulatory sequences at the selected loci, regardless of the sample group. Statistically significant differences at the level of CpG methylation were observed only in case of the *HCK* gene. The *HCK* locus contains a CpG island in the region of its promoter. Therefore, possible mechanism occurs in which *HCK* expression is modulated by epigenetic modifications such as DNA methylation. The obtained results indicate a reduced level of *HCK* methylation in the mammary gland secretory tissue of cows diagnosed with chronic mastitis and infected with coagulase-positive staphylococci. This may be related to previously reported overexpression of this gene during the course of mastitis (Kościuczuk et al., 2017). The hematopoietic cell kinase, a member of the Src family kinases, is associated with functions such as adhesion and migration, phagocytosis, formation of membrane protrusions, lysosome exocytosis, podosome formation and actin polymerization (Guiet et al., 2008). HCK activation is launched by a variety of stimulants, including binding of bacterial lipopolysaccharides to the CD14/Toll-like receptor protein complex or cytokines such as IL-2 and IL-6. After activation, HCK engages a range of downstream signaling pathways that mediate various aspects of the immune response (Poh et al., 2015).

Many components have been identified in milk that are associated with the non-specific immune response. These include cellular defense components, e.g. pro-inflammatory cytokines or lactoferrin, acute phase proteins, and a wide range of antimicrobial peptides and proteins. Many of these components come from specialized

defense cells that penetrate the site of inflammation. The obtained data suggest that increased expression of the *HCK* gene in the group of cows diagnosed with chronic mastitis may be related to the observed decreased level of methylation, which in turn can affect the inflammation process during the course of the disease.

Gene expression is a complex process regulated not only through DNA methylation, but also by various other mechanisms. In addition to cytosine methylation, histone proteins and their post-translational modifications, and complexes that remodel chromatin and various noncoding RNA, also play central roles in the establishment of stable gene expression patterns (Gibney and Nolan, 2010). In the context of a complex network of epigenetic modifications, differences in gene expression in response to the methylation profiles of some genes may be negligible. In the case of the analyzed loci, the observed low methylation level seems not to correspond to the gene activity level. However, the lack of differences between the studied groups (CoPS, CoNS, H) may indicate that mechanisms other than DNA methylation are involved in controlling mRNA expression of the analyzed genes.

In a similar study performed with the same biological material, Bagnicka et al. (2021) used next generation sequencing (NGS) to identify a total of 44 microRNAs with differential expression in the udder tissues of cows infected with *S. aureus,* compared to a control group. The microRNA target genes identified as having differential expression were also found to be linked to KEGG pathways and GO categories involved in processes associated with bacterial infection caused by various pathogens. Furthermore, 20 miRNAs (CoPSvsH) were defined that take part in the immune processes, and 622 related target genes were identified. It is therefore feasible that the genes analyzed here are regulated not by DNA methylation, but through other mechanisms, such as the action of miRNA molecules. The observed overexpression of these loci in mastitic cows may result from a low or high expression level of a given miRNA. However, this statement needs to be confirmed by further research.

The current literature data on DNA methylation and its role in cow mastitis are limited, but the available results indicate that DNA methylation plays a role in susceptibility to mastitis in cattle. Vanselow et al. (2006) reported that the DNA promoter region for the alphaS1-casein gene can be hypomethylated in the bovine mammary gland. During *E. coli* infection, this region is methylated at three CpG dinucleotides. Similar results were obtained for udder infection with *Streptococcus uberis*. Methylation level at three CpG sites for alphaS1-casein was much higher in the mastitic udder tissue (range of 28 to 68%) than in the control tissue (range of 10 to 25%) (Swanson et al., 2009; Singh et al., 2010). Studies also showed increased methylation level of the *CD4* gene promoter in the whole blood of mastitic cows compared to control cows. Moreover, the DNA methylation level was negatively correlated with the *CD4* gene expression level. The authors suggest that the DNA methylation status of the bovine *CD4* gene may be a promising epigenetic marker for selection against mastitis (Wang et al., 2013). Mao et al. (2015) determined methylation level in the CpG island of exon 1 of the *CXCR1* gene in the bovine mammary gland secretory tissue infected with *S. aureus,* and concluded that the observed lower methylation level of CpG site at position 519 may regulate *CXCR1* expression in mastitic cows. In turn, Song et al. (2016), using a MeDIP-chip technique to obtain the DNA methylation profile of bovine peripheral blood lymphocytes from healthy (CK) and mastitic cows infected with *S. aureus* (SA), identified 1078 differentially methylated regions (DMRs). After comparing transcriptome analysis data with the results of DNA methylation analysis, they confirmed the presence of 58 genes, of which 20.7% of distinctly hypermethylated loci showed down-regulated expression in the SA group compared to the CK group, while 14.3% of hypomethylated genes showed up-regulated expression. Further functional analysis suggested that these genes were related to inflammation, the ErbB signaling pathway and DNA repair. Three genes, *NRG1, MST1* and *NAT9*, were strongly correlated with the progression of subclinical mastitis, and could be used as biomarkers for the improvement of bovine mastitis resistance. What is more, the most recent study by Ju et al. (2020) revealed the presence of 494 DMRs in bovine peripheral blood neutrophils in mastitic cows infected with *E. coli* (MC). The level of methylation in mastitic cows was lower than in healthy cows (HC). In addition, based on transcriptome sequencing and RRBS library data, they identified 29 differentially expressed genes with DMRs. The identified genes included *CITED2*, *SLC40A1* and *LGR4* which, as the authors suggest, can be used as candidate genes for studies of cow mastitis induced by *E. coli*.

The studies reported above have proven that epigenetic changes are related to mastitis, and advance our understanding of the role of DNA methylation in the mastitis development process. Moreover, they provide information about new target genes and epigenetic markers for the prevention of mastitis and developing mastitis resistance in dairy cattle.

Conclusions

In this study, we determined DNA methylation levels of potential promoter regions of the bovine CCL2*, IL18, HCK, F11R, CD8A, PDIA3, LGMN, HSPA1A* and *NFKBIA* genes, in the mammary gland secretory tissue of cows infected with coagulase-positive or coagulase-negative staphylococci. The obtained data support a potential involvement of DNA methylation in the regulation of *HCK* gene expression, associated with the course of mastitis. The results of this stage of research also indicate that lack of variation between test groups and control cows in CpG methylation at the rest of the analyzed loci seems not to correspond to mastitis-associated differential expression of these genes. It is therefore feasible that,

in chronic mastitis, the genes analyzed here are regulated not by DNA methylation, but through other mechanisms.

Conflict of interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The study was approved by the III Local Ethical Committee in Warsaw (Approval 15/2010). Animals were maintained on the Experimental Farm at the Institute of Genetics and Animal Biotechnology in Jastrzębiec, near Warsaw, Poland. The Experimental Farm is an integral part of the institute, and maintains a herd of 120 dairy cows of the Holstein-Friesian breed. The owner of the herd gave written permission regarding the use of the cows in this study.

Authorship

E. Semik-Gurgul and T. Ząbek designed and conducted the study, analyzed and interpreted data and wrote the manuscript; E. Kawecka-Grochocka and M. Zalewska performed the DNA sample isolation. E. Kościuczuk performed microbiological analysis and the sample collection; E. Bagnicka conceived the idea and contributed to study execution. All authors gave their final approval of the manuscript.

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Received: 25 III 2021
Accepted: 29 VII 2021