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POLYMORPHISM IN *BMAP-27* AND *BMAP-28* GENES AND THEIR RELATIONSHIP WITH MILK PRODUCTION TRAITS IN CATTLE

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Abstract. The study aimed to analyze polymorphisms located in exon 4 of the *CATHL5* gene encoding the BMAP-28 protein and in exon 4 of the *CATHL6* gene encoding the BMAP-27 protein concerning milk performance parameters, such as milk yield, fat, protein, and lactose content and somatic cell count in milk. The PCR-RFLP method using the ACRS technique was used in the study to create a cleavage site for enzymes. Based on the results of genotyping, results for individual SNPs were obtained, with statistically significant differences at the $P \leq 0.05$ and $P \leq 0.01$. Between individual genotypes of the studied polymorphisms and selected traits of milk production.

Key words: *CATHL5*, *CATHL6*, dairy cattle, single nucleotide polymorphism.

INTRODUCTION

Livestock breeding entails the need to search for selection strategies and ways to raise the standards of keeping animals to improve their genetic and non-genetic conditions, whit which it is possible to increase the efficiency of the raw materials obtained. Cows are productive animals whose breeding has two main directions: one is dairy cattle, and the other is beef cattle. According to the Polish Federation of Cattle Breeders and Milk Producers (PFHBiPM 2021), at the end of 2021, the number of dairy cattle was 6,371.5 thousand. According to the assessment of milk performance of cows under assessment – about 40% of the dairy cattle population – carried out by PFHBiPM, the milk obtained with an average yield of 8837 kg of milk per head is characterized by the parameters of the average protein content of 3.42% and fat content of 4.13%. Lactation in cows is greatly influenced by the following factors: genetics, as well as the health and immunity of animals, as well as nutrition, the way of keeping, or the method of milking (Kołacz et al. 2020). One of the most common reasons for reduced milk production in cows is mastitis, which is associated with infection by microorganisms that cause mastitis, where poor hygiene conditions on the farm often contribute to the spread of infection in the herd (1). An important direction of research undertaken to reduce the susceptibility to infection with mastitis-causing pathogens is the search for individuals that genetically have increased immunity and natural defense mechanisms against infection (Kurz et al. 2019). Hence, there is a growing need to search for naturally occurring proteins in the cow's body, which could be the target of marker-assisted selection. So far, much attention has been paid to a group of proteins belonging to antimicrobial peptides (Antimicrobial Peptides, AMP), which include cathelicidins. These proteins are known in many species of ani-

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mals, including mammals, amphibians, and reptiles (Hemshekhar et al. 2016). Cathelicidins have antibacterial, antiviral, antifungal, and immunomodulatory properties (Kościuczuk et al. 2012). In addition, studies are being carried out showing the impact of genes from the cathelicidin family on animal performance parameters (Tomasinsig et al. 2010; Seo et al. 2016; Mei et al. 2019).

BMAP proteins (bovine myeloid antimicrobial peptides) can be distinguished among the bovine cathelicidins. These proteins have an α-helix structure, the most commonly observed type of cathelicidin structure in mammals. It is also assumed that the structure of the α-helix is also a prototype, based on which other structural systems of other cathelicidins were shaped, and this evolution of proteins in individual organisms may be associated with contact with factors such as pathogens. In cattle, the BMAP-27 and BMAP-28 proteins encoded by the *CATHL6* and *CATHL5* genes, respectively, can be distinguished (Wódz and Brzezińska-Błaszczyk 2015). Each gene is located on the 22nd chromosome of cattle and consists of four exons, between which there are three introns. Cathelicidin precursor proteins have an N-terminal signal peptide, a cathelin domain, and a C-terminal variable region. The first 3 exons encode the signal peptide and the cathelin domain, while the 4th exon encodes the variable region, which is responsible for the main functions of cathelicidins (Agier et al. 2015). Expression of genes encoding BMAP proteins has been observed in various cells of bovine tissues, including the ovary, oviduct, uterus, rumen, large intestine, liver, spleen, lymph nodes, peripheral blood, lungs, and mammary gland (Whelehan et al. 2014).

The study searched for possible relationships between polymorphisms mapped within the *CATHL5* and *CATHL6* genes and milk performance parameters in Polish Holstein-Friesian black-and-white cattle. Considering the possible role of bovine cathelicidins in the body, an analysis of the impact of polymorphisms within the *CATHL5* and *CATHL6* genes was carried out concerning performance parameters, including milk yield, protein content and yield, fat content and yield, lactose content and yield and somatic cell count in milk. Due to the coding of the variable region, polymorphisms mapped in exon 4 were selected.

MATERIALS AND METHODS

The study covered a herd of Polish Holstein-Friesian dairy cattle of the black-and-white variety bred in the Opolskie Voivodeship. The herd consisted of 279 cows, which were kept using the accessible stall system. Feeding was performed using a Total Mixed Ration (TXM) system. The herd under study was milked using a mechanical milking machine twice a day. The herd had documented data on milk performance in connection with the assessment conducted by the Polish Federation of Cattle Breeders and Dairy Producers.

Blood collected from the zygomatic vein of each cow included in the study was used as the research material. Blood was collected in sterile tubes that contained K_3EDTA, an anticoagulant. DNA was then isolated from the samples using the commercial MasterPure Kit (Lucigen) according to the manufacturer's protocol.

The tests were carried out on the test herd following Resolution No. 13/2016 of the National Ethical Committee for Animal Experiments on Genotyping and Marking of Animals.

The study used the PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) technique, for which primers were designed based on data in the Ensmbl database, also using the ACRS (Amplification Created Restriction Site) method. Using the NCBI (National Center of Biotechnology Information) database, the sequence of the *CATHL5* and *CATHL6* genes was searched. Then, the Ensmbl database was used to design the primers, where information on existing single nucleotide polymorphisms (SNPs) was searched. Based on the gene sequences, primers were designed using the Primer3 tool. Subsequently, the prepared sequence with the selected SNP sites was analyzed in the NEBcutter program to select restriction enzymes. Based on the analysis, the ACRS method was used to create cleavage sites for individual enzymes. After

designing the primers and matching the restriction enzymes, virtual digestion was performed in the RestrictionMapper program, thus obtaining data on the expected digestion products. Data on selected SNPs, primer sequences, primer amplification temperatures, product sizes, and selected restriction enzymes are presented in Table 1.

Table 1. SNPs, primers, product and restriction enzymes data

Gene	SNP	rs	Primers	Temp.	Product size	Restriction enzyme
CATHL5	2380 *T>C*	437338944	F: AGTTTGTGGGGATGTCCAAG R: GAGGGCCTCCCAGAGTAAC	52°C	351 bp	*Taq*I
CATHL6	2375 *T>A*	432444224	F: TTCTGGTTCATAGTTGCACAG R: GATCACGCACACACCAAAAC	58°C	318 bp	*Dra*I
	2364 *T>G*	466104221				*Tsp*RI
	2372 *G>C*	454241760	F: ATAGTTGCAGAGTGTTGGTA R: GATCACGCACACACCAAAAC	56°C	309 bp	*Rsa*I

The PCR reaction was carried out according to standard conditions using a reaction mixture consisting of a reaction buffer (A&A Biotechnology), specific primers for individual polymorphisms, and molecularly pure water. The reactions were carried out according to the standard optimization procedure, consisting of initial denaturation for 5 min at 95°C, then successively in 30 cycles: denaturation – 30 seconds at 95°C; attaching primers – 45 seconds at dedicated temperatures for primers; DNA chain extension – 30 seconds at 72°C followed by final extension for 8 minutes at 72°C.

Genotyping of the amplification products was performed using the PCR-RFLP method. The products were incubated at the enzyme's temperature and time specified by the manufacturer. After incubation, the resulting digests were separated on a 3% agarose gel to evaluate the resulting genotypes.

Based on the obtained results, a statistical analysis was carried out between the polymorphisms: *CATHL5/Taq*I, *CATHL6/Dra*I, *CATHL6/Tsp*RI, and *CATHL6/Rsa*I and milk performance parameters using the Statistica 12 program (StatSoft, Inc.). Mean values ($\bar{x}$) and standard deviation (SD) for the compared relationships were calculated, and Duncan's multiple range test was used for a one-way analysis of variance. The analyzed milk parameters included: milk yield, protein content, fat content, fat-to-protein ratio, lactose content, and somatic cell count. To meet the conditions of normal distribution, the number of SCC somatic cells expressed in thousands/ml of milk was transformed into the natural logarithm of LnSCC, according to Ali and Shook (1980). The Kruskall–Wallis test was performed to assess the significance of differences in the mean somatic cell count.

RESULTS

Based on the studies, three possible genotypes for the *CATHL5/Taq*I polymorphism were identified, and the frequencies for individual genotypes and alleles are presented in Table 2.

Table 2. The genotype and allele frequencies of the studied *CATHL5* SNP

SNP	Number of cows	Genotype frequencies		Allele frequencies	
CATHL5/*Taq*I	142	*GG*	0.509	*G*	0.720
	118	*GA*	0.423		
	19	*AA*	0.068	*A*	0.280

The results of the statistical analysis for the genotype and milk performance parameters are presented in Table 3. The study showed a statistically significant difference ($P \leq 0.05$) in the case of milk fat content in cows with the *AA* genotype, which had the highest value of this parameter.

Table 3. Mean values and standard deviation of studied traits in references to *CATHL5*/*Taq*I genotypes

Genotype	Milk yield [kg]	Fat content [%]	Protein content [%]	Lactose content [%]	LnSCC
GG	33.78 ± 8.11	3.81[a] ± 0.71	3.51 ± 0.34	4.92 ± 0.16	4.23 ± 0.84
GA	33.49 ± 7.76	3.87 ± 0.76	3.53 ± 0.34	4.93 ± 0.16	4.22 ± 0.81
AA	34.46 ± 7.38	3.91[a] ± 0.66	3.53 ± 0.27	4.94 ± 0.15	4.23 ± 0.83

[a] Values in columns with different letters differ significantly (P ≤ 0.05).

Studies involving polymorphisms in the *CATHL6* gene identified all three possible genotypes for all tested SNPs. Individual frequencies for genotypes and alleles are presented in Table 4.

Table 4. The genotype and allele frequencies of the studied *CATHL6* SNP

SNP	Number of cows	Genotype frequencies		Allele frequencies	
CATHL6/*Dra*I	233	*TT*	0.835	*T*	0.907
	40	*TA*	0.143	*A*	0.093
	6	*AA*	0.022		
CATHL6/*Tsp*RI	39	*TT*	0.140	*T*	0.554
	231	*TG*	0.828	*G*	0.446
	9	*GG*	0.032		
CATHL6/*Rsa*I	89	*GG*	0.319	*G*	0.636
	177	*GC*	0.634	*C*	0.364
	13	*CC*	0.047		

In the case of the *CATHL6/Dra*I polymorphism, it was observed that the milk of cows with the *TT* genotype had the highest lactose content and the lowest in cows with the *AA* genotype, the difference between the results was statistically significant (P ≤ 0.01).

For the *CATHL6/Tsp*RI polymorphism, it was noted that in the case of milk yield, the highest value of this parameter was found in cows with the *GG* genotype (P ≤ 0.01), while the highest fat and protein content in milk was observed in cows with the *TT* genotype (P ≤ 0.01). In the case of somatic cells, it was observed that the lowest number of somatic cells is found in heterozygous cows and the highest in cows with the *GG* genotype (P ≤ 0.01).

Analyzing the results obtained for *CATHL6/Rsa*I, the highest milk yield was observed in cows with the *GG* genotype (P ≤ 0.01). The highest protein content was observed in *CC* genotype cows (P ≤ 0.05). The lowest number of somatic cells was found in cows with the *GG* genotype (P ≤ 0.01).

Table 5 presents the statistical analysis results of the relationship between the milk yield parameters and the obtained genotypes, marking all statistically significant differences.

Table 5. Mean values and standard deviation of studied traits in references to *CATHL6* genotypes

Genotype	Milk [kg]	Fat content [%]	Protein content [%]	Lactose content [%]	LnSCC
			CATHL6/*Dra*I		
TT	33.56 ± 7.75	3.84 ± 0.72	3.52 ± 0.33	4.93[A] ± 0.15	4.20 ± 0.80
TA	34.28 ± 8.27	3.85 ± 0.76	3.53 ± 0.34	4.90[B] ± 0.17	4.33 ± 0.89
AA	34.62 ± 10.88	3.85 ± 0.64	3.47 ± 0.30	4.85[AB] ± 0.12	4.23 ± 1.02
			CATHL6/*Tsp*RI		
TT	34.32 ± 8.72	3.93B ± 0.74	3.55[D] ± 0.35	4.91 ± 0.17	4.22[bG] ± 0.85
TG	33.47[A] ± 7.67	3.84C ± 0.73	3.52[E] ± 0.34	4.93 ± 0.16	4.20[bH] ± 0.82
GG	35.42[A] ± 8.87	3.60BC ± 0.72	3.40[DE] ± 0.28	4.84 ± 0.18	4.52[GH]± 0.81
			CATHL6/*Rsa*I		
CC	31.63[AB] ± 6.96	3.78 ± 0.71	3.55[a] ± 0.34	4.90[dC] ± 0.16	4.33[DE] ± 0.75
GC	33.70[B] ± 8.17	3.87 ± 0.76	3.53 ± 0.34	4.92[d] ± 0.16	4.24[E] ± 0.83
GG	34.02[A] ± 7.52	3.80 ± 0.67	3.50[a] ± 0.33	4.94[C] ± 0.17	4.17[D] ± 0.83

[a–d] Values in columns with different letters differ significantly (P ≤ 0.05).

[A–H] Values in columns with different letters differ significantly (P ≤ 0.01).

DISCUSSION

Cathelicidins belong to proteins belonging to antimicrobial peptides leads to the search for directions that could help raise the level of cattle breeding. Cathelicidins have a broad spectrum of activity, including antibacterial, and are involved in angiogenesis, neutralization of toxins, and iron metabolism, their participation in reproduction has been observed (Avila 2017), and they play a considerable role in immune mechanisms (Paget 2014). Their antimicrobial properties are a good indicator of a path that could lead to the selection of individuals with a gene variant that favors the prevention and possible control of infection with bacteria that cause mastitis. Their role in connection with milk performance characteristics such as milk yield, fat, protein, and lactose content is constantly being explored, and based on the results obtained, it can be concluded that there are relationships between polymorphisms and individual characteristics.

In 1996, Skerlavaj et al. (1996) conducted studies aimed at evaluating the antimicrobial properties of proteins: BMAP-27 and BMAP-28, encoded by *CATHL6* and *CATHL5*, respectively, where they obtained results indicating antibacterial activity against bacteria that are identified during mastitis.

Research by Giagu et al. (2022) showed that identifying cathelicidins in milk is an excellent marker for identifying ongoing mastitis in cows.

Similar studies, considering the possibility of using cathelicidins as a marker allowing the identification of subclinical or clinical mastitis, were conducted by Wollowski (2022).

For studies relating to somatic cell counts, Wu et al. (2015) conducted studies between the C86G polymorphism within the *CATHL5* gene encoding the BMAP-28 protein, in which they showed a statistically significantly lower number of somatic cells in the case of the *CC* genotype, and higher protein content and higher fat content for the heterozygous genotype, the results were statistically significant ($P \leq 0.05$).

Our research showed statistically significant differences between the examined polymorphisms within the *CATHL5* and *CATHL6* genes and all milk performance traits assessed in this study. For the *CATHL5/Taq*I polymorphism, the highest milk lactose content was observed for the *AA* genotype. In the case of *CATHL6/Dra*I, the highest lactose content was observed for the *TT* genotype. In turn, examining the *CATHL6/Tsp*RI polymorphism, the highest milk yield was observed for the *GG* genotype, the highest fat and protein content, and the lowest somatic cell count for the *TT* genotype. The *GG* genotype obtained for the *CATHL6/Rsa*I polymorphism showed the highest milk yield, lactose content, and the lowest somatic cell count. All results were statistically significant. Since mastitis significantly reduces the yield and quality of milk obtained, it can be seen that based on the results, there is a tendency to obtain high milk yield with higher content of fat, protein, and lactose while reducing the number of somatic cells, which may indicate an indirect effect of cathelicidins on milk performance parameters by supporting the immune system and acting against pathogens causing mastitis. However, further comprehensive research is needed to confirm the existence of such a relationship.

CONCLUSIONS

Research is needed to improve dairy farming, and mastitis is the main problem causing breeding and milk quality difficulties. Research into the possibility of using marker-assisted selection is essential. Due to their characteristics and functions, cathelicidins are an interesting subject for deepening research to understand the relationship between polymorphisms and parameters of milk performance. The studies showed statistically significant differences for individual genotypes in all polymorphisms being the subject of the study. Noteworthy is the statistical significance observed for the parameter, which is the number of somatic cells. At the same time,

based on preliminary research, it can be concluded that some genotypes correlate with individual parameters of milk performance.

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POLIMORFIZM W GENACH *BMAP-27* I *BMAP-28* ORAZ ICH ZWIĄZEK Z CECHAMI PRODUKCJI MLEKA U BYDŁA

Streszczenie. Celem pracy była analiza polimorfizmów zlokalizowanych w eksonie 4 genu *CATHL5* kodującego białko BMAP-28 oraz w eksonie 4 genu *CATHL6* kodującego białko BMAP-27 w odniesieniu do parametrów użytkowości mlecznej, takich jak: wydajność mleka, zawartość tłuszczu, białka i laktozy oraz liczba komórek somatycznych w mleku. W badaniach użyto metody PCR-RFLP z wykorzystaniem techniki ACRS do wykreowania miejsca cięcia dla enzymów. Na podstawie wyników genotypowania uzyskano wyniki dla poszczególnych SNP, w których istnieją statystycznie istotne różnice na poziomie $P \leq 0,05$ oraz $P \leq 0,01$ pomiędzy poszczególnymi genotypami badanych polimorfizmów a wybranymi cechami użytkowości mlecznej.

Słowa kluczowe: *CATHL5, CATHL6*, bydło mleczne, polimorfizm pojedynczego nukleotydu.