ACTA SCIENTIARUM POLONORUM

Acta Sci. Pol. Zootechnica 21(4) 2022, 17–22

www.asp.zut.edu.pl pISSN 1644-0714 eISSN 2300-6145 DOI:10.21005/asp.2022.21.4.02

ORIGINAL PAPER

Received: 18.12.2022
Accepted: 13.01.2023

IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISM WITHIN BACTENCIN-5 AND BACTENCIN-7 CODING GENES IN ASSOCIATION WITH MILK PRODUCTION TRAITS

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ABSTRACT

Bactencins belong to the bovine cathelicidin family of proteins, and their role in the body is significant. The study analyzed the relationship between polymorphisms within the *CATHL2* and *CATHL3* genes encoding the Bac-5 and Bac-7 proteins. The study included a herd of Polish Holstein-Friesian cows of the black-and-white breed. Tests were conducted using the PCR-RFLP method with the introduction of the ACRS modification. The study showed that different genetic variants within the polymorphisms studied were significantly associated with selected parameters of milk performance, such as milk yield ($P \leq 0.01$), fat ($P \leq 0.01$), protein ($P \leq 0.01$ and $P \leq 0.05$) and lactose ($P \leq 0.01$ and $P \leq 0.05$) content and somatic cell count ($P \leq 0.01$ and $P \leq 0.05$).

Key words: *CATHL2*, *CATHL3*, dairy cattle, SNP

INTRODUCTION

Immune peptides play an essential role in maintaining the healthy balance of livestock. Classic endogenous antimicrobial peptides in cattle include cathelicidins and defensins. Cathelicidins have shown great importance in participating in biological activity in cattle through their antimicrobial, proinflammatory, or immunomodulatory functions [Deptuła et al. 2019]. Among bovine cathelicidins, the most characterized group are the proline- and arginine-rich peptides found among others in neutrophils – the bactencins [Tomasinsig et al. 2010]. Their name is indirectly related to their primary function – bactericidal. They show vigorous activity against Gram-positive and Gram-negative bacteria by destroying the cell membrane and organelles of bacteria [Agier and Brzezińska-Błaszczyk 2016]. Among the bactencins, we can distinguish bactencin 5 (Bac-5) and bactencin 7 (Bac-7), encoded by the *CATHL2* and *CATHL3* genes, respectively. The genes encoding bovine cathelicidins are located on chromosome 22 and are composed of 4 exons separated by 3 introns. Exon 4 is responsible for encoding the variable region of the protein, which is responsible for the function of the peptide [Kościuczuk et al. 2012].

Cathelicidins are found in many animal species: mammals, including cattle, pigs, sheep, goats, mice, monkeys, or humans, as well as in birds or reptiles [Avila 2017]. Their importance in organisms is significant, and various lines of research have been undertaken to link these proteins to their role in animal husbandry. Their function in the immune system is unquestionably the main issue analyzed by various research teams; however, the effect of cathelicidins – direct or indirect – on performance parameters has attracted increasing interest in recent years [Cubeddu et al. 2017].

Finding the relationship between cathelicidins and mastitis in cattle is an essential research subject. Mastitis is one of the main factors adversely affecting dairy cattle breeding, causing losses [Royster and Wagner 2015]. Expression of genes encoding cathelicidins has been shown, among other things, in the udder cells of cows and the milk itself [Whelehan et al. 2014]. A link has also been observed with increased cathelicidins in cases of diagnosed subclinical and clinical mastitis, indicating

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increased protein production after contact with pathogens [Smoleński et al. 2011]. This fact has led to increasing research into a test that identifies these proteins in milk, which could quickly detect potentially sick cows [Addis et al. 2017].

The role played by cathelicidins in the antimicrobial and anti-inflammatory process in the mammary gland in cows leads to the assumption that these proteins may show a connection with the number of somatic cells and indirectly affect other parameters of dairy performance, such as yield, fat, protein and lactose content.

The purpose of the research was to analyze possible relationships between selected polymorphisms in the genes *CATHL2* and *CATHL3* and selected parameters of dairy performance.

MATERIAL AND METHODS

The material for the study consisted of blood drawn from cows from a herd of Polish Holstein-Friesian black-and-white dairy cattle, numbering 279 individuals. The herd was maintained using a free-stall system, and feeding was carried out using a total mixed ration system. Milking was carried out twice a day using a mechanical milking machine. The herd included in the study was under the evaluation of the Polish Federation of Cattle Breeders and Milk Producers and had documented data on milking performance. The material for the study was collected during the summer, and none of the cows showed a clinical course of mastitis. Blood was collected from the zygomatic vein into a sterile tube containing K3 EDTA. DNA isolation was performed using a commercial MasterPure Kit (Lucigen) according to the manufacturer's protocol.

PCR-RFLP (Polymerase Chain Reaction – Restriction Fragment Length Polymorphism) genotyped the selected polymorphisms. Using the Amplification Created Restriction Site (ACRS) method, primers were designed based on *CATHL2* and *CATHL3* gene sequence data from databases: Ensmbl and NCBI National Center of Biotechnology Information. The primers were designed using the Primer 3 tool, and the prepared sequence was analyzed in NEBCutter software to select cutting sites and restriction enzymes. After designing the array, a virtual analysis was performed in RestrictionMapper. Data on selected SNPs, primer sequences, primer amplification temperatures, product size, and selected restriction enzymes are shown in Table 1.

The composition of the reaction mixture for the amplification reaction carried out for each polymorphism tested was a ready-to-use mix for PCR (A&A Biotechnology), a pair of specific primers, and water. The reaction profile was carried out according to a standard procedure: pre-denaturation for 5 min at 95°C, followed sequentially by 30 cycles: denaturation – 30 seconds at 95°C; primer attachment – 45 seconds at dedicated primer temperatures; DNA chain extension – 30 seconds at 72°C, followed by final extension for 8 minutes at 72°C.

Once the products were obtained, they were incubated at the restriction enzyme's dedicated temperature and time specified by the manufacturer. The digestion products were then separated in a 3% agarose gel to visualize and evaluate the obtained genotypes.

Based on the genotyping results, statistical analysis was carried out between the polymorphisms studied and milk performance parameters using Statistica 12 software [StatSoft 2013]. Mean values ($\bar{x}$) and standard deviation (SD) for the compared relationships were calculated, and Duncan's multiple range test was used for univariate analysis of variance. The dairy parameters analyzed were as follows: milk yield, protein content, fat content, lactose content, and somatic cell count. To meet the conditions of normal distribution, the number of somatic cells SCC expressed in thousands per ml of milk was transformed into the natural logarithm of LnSCC, according to Ali and Shook [1980]. The Kruskal-Wallis test was performed to assess the significance of differences in mean somatic cell count. For statistical analysis, the following linear model formula was used:

$$Y_{ij} = m + a_i + e_{ij}$$

where:

Y_{ij} – trait level,
m – mean value,
a_i – fixed effect of genotype,
e_{ij} – random error.

RESULTS

In studies involving polymorphisms in the *CATHL2* gene, all three possible genotypes were identified for all SNPs studied. Individual frequencies for genotypes and alleles are shown in Table 2.

For the *CATHL2/Hha*I polymorphism, we observed the highest milk yield in cows with the *TT* genotype ($P \leq 0.01$), the highest lactose content in milk from cows with the *CC* genotype ($P \leq 0.01$), and the lowest somatic cell count in milk from *TC* cows ($P \leq 0.05$).

For the *CATHL2/Dde*I polymorphism, the highest milk fat content was observed in *AA* and *AT* cows, the highest milk protein content was observed in *AT* cows ($P \leq 0.01$), and cows with the *AA* genotype had the highest milk lactose content ($P \leq 0.01$).

For the *CATHL2/Rsa*I polymorphism, the highest fat content in milk was observed in *GG* cows ($P \leq 0.01$), the highest lactose content in milk was observed in cows with the *CC* genotype ($P \leq 0.01$), and heterozygous cows had the lowest somatic cell count ($P \leq 0.01$).

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

Gene	SNP	rs	Primers	Temp.	Product size	Restriction enzyme
CATHL2	1843 *T>C*	457785915	F:TGGTTCACAGCTTCAGCG R: AGTTGGAAGAGGCGAGACAA	50°C	324 bp	*Hha*I
	1963 *A>T*	716076411				*Dde*I
	1856 *G>C*	801306179	F:GGTCCCTAAGGTTCCTGGAG R: GGTGGACGACGGATTGGTGTA	53°C	154 bp	*Rsa*I
CATHL3	2361 *T>G*	458005961	F: TTGTCATGGTTTACAGCTTCACA R: TGGGGGAAAGTTGTCTTCAC	50°C	458 bp	*Tsp*RI
	2512 *C>T*	800945414				*Nla*III
	2448 *T>G*	1115755318	F: CCTGGGCCAAGGCCGAT R: TGGGGGAAAGTTGTCTTCAC	51°C	364 bp	*Hin*fI

rs - Reference SNP cluster ID

Table 2. The genotype and allele frequencies of the studied *CATHL2* SNPs

	N	Genotype frequencies		Allele frequencies	
***CATHL2/Hha*I**	153	*TT*	0.548	*T*	0.749
	112	*TC*	0.401	*C*	0.251
	14	*CC*	0.050		
***CATHL2/Dde*I**	97	*AA*	0.348	*A*	0.647
	167	*AT*	0.599	*T*	0.353
	15	*TT*	0.054		
***CATHL2/Rsa*I**	31	*GG*	0.111	*G*	0.518
	227	*GC*	0.814	*C*	0.482
	21	*CC*	0.075		

N – number of cows

Table 3. Mean values and standard deviation of studied traits in references to *CATHL2* genotypes

Genotype	Milk yield, kg	Fat content, %	Protein content, %	Lactose content, %	LnSCC
			***CATHL2/Hha*I**		
TT	34.16^{A}±7.86	3.83±0.72	3.50±0.34	4.92^{A}±0.16	4.24±0.82
TC	33.27±8.03	3.87±0.76	3.54±0.35	4.93^{B}±0.17	4.19^{a}±0.80
CC	32.16^{A}±7.43	3.80±0.59	3.54±0.28	4.98^{AB}±0.13	4.34^{a}±1.04
			***CATHL2/Dde*I**		
AA	33.59±8.10	3.87^{A}±0.72	3.52^{A}±0.33	4.94^{A}±0.17	4.24±0.83
AT	33.63±7.89	3.87^{B}±0.73	3.53^{B}±0.35	4.92^{A}±0.16	4.21±0.83
TT	35.32±7.08	3.40^{AB}±0.69	3.42^{AB}±0.28	4.89^{AB}±0.18	4.25±0.76
			***CATHL2/Rsa*I**		
GG	34.15±9.22	3.89^{A}±0.73	3.50±0.39	4.92^{A}±0.17	4.32^{a}±0.85
GC	33.56±7.65	3.85^{B}±0.73	3.53^{A}±0.33	4.92^{a}±0.16	4.20^{a}±0.82
CC	34.41±7.92	3.64^{AB}±0.69	3.46^{A}±0.27	4.95^{Aa}±0.17	4.24±0.89

$^{a, b}$ – values in columns with different letters differ significantly ($P \leq 0.05$).
$^{A, B}$ – values in columns with different letters differ significantly ($P \leq 0.01$).

In studies involving polymorphisms in the *CATHL3* gene, all three genotypes were identified for all SNPs studied. Individual frequencies for genotypes and alleles are shown in Table 4.

Upon analyzing the results obtained for the *CATHL3/Tsp*RI polymorphism, we found the highest protein content in the milk obtained from cows with the

Table 4. The genotype and allele frequencies of the studied *CATHL3* SNPs

	N	Genotype frequencies		Allele frequencies	
***CATHL3/Tsp*RI**	123	*TT*	0.441	*C*	0.695
	142	*TG*	0.509	*G*	0.305
	14	*GG*	0.050		
***CATHL3/Nla*III**	180	*CC*	0.645	*C*	0.794
	83	*CT*	0.297	*G*	0.206
	16	*TT*	0.057		
***CATHL3/Hin*fI**	170	*TT*	0.609	*C*	0.778
	94	*TG*	0.337	*G*	0.222
	15	*GG*	0.054		

N – number of cows

Table 5. Mean values and standard deviation of studied traits in references to *CATHL3* genotypes

Genotype	Milk yield, kg	Fat content, %	Protein content, %	Lactose content, %	LnSCC
			***CATHL3/Tsp*RI**		
TT	33.46±7.99	3.80±0.71	3.52 ± 0.34^{a}	$4.91^{a}\pm0.17$	4.26±0.86
TG	33.92±7.94	3.87±0.74	3.51 ± 0.34^{b}	$4.94^{a}\pm0.16$	4.20±0.80
GG	33.79±7.20	3.88±0.81	3.57 ± 0.33^{ab}	4.92±0.14	4.14±0.73
			***CATHL3/Nla*III**		
CC	33.53±7.78	3.87±0.71	$3.53^{A}\pm0.34$	$4.94^{a}\pm0.17$	4.22±0.79
CT	34.03±8.07	3.82±0.79	$3.51^{B}\pm0.34$	$4.92^{Aa}\pm0.16$	4.21±0.85
TT	33.83±8.80	3.66±0.60	$3.47^{AB}\pm0.31$	$4.89^{A}\pm0.18$	4.28±1.10
			***CATHL3/Hin*fI**		
TT	$33.93^{A}\pm8.07$	3.83±0.73	$3.52^{a}\pm0.34$	4.92±0.16	4.25±0.83
TG	$32.96^{A}\pm7.72$	3.87±0.75	$3.53^{b}\pm0.33$	4.93±0.17	4.18±0.82
GG	$35.81^{AB}\pm7.42$	3.85±0.57	$3.47^{ab}\pm0.34$	4.94±0.16	4.23±0.79

$^{a, b}$ – values in columns with different letters differ significantly ($P \leq 0.05$).
$^{A, B}$ – values in columns with different letters differ significantly ($P \leq 0.01$).

GG genotype ($P \leq 0.01$), whereas the highest lactose content was observed in heterozygotes ($P \leq 0.05$).

For the *CATHL3/Nla*III polymorphism, the highest protein and lactose content was recorded in milk from cows with the *CC* genotype ($P \leq 0.05$).

For the *CATHL3/Hin*fI polymorphism, the highest milk yield was observed in cows with the *GG* genotype ($P \leq 0.01$), and the highest protein content was observed in heterozygotes ($P \leq 0.05$).

DISCUSSION

Studies involving association analysis between single nucleotide polymorphisms and milk performance parameters in cattle have been of interest to researchers for many years [Suchocki et al. 2022].

Relationships between somatic cell counts and polymorphisms within cattle genes have been analyzed extensively, and there is a constant need to improve knowledge to improve health in dairy cows, which translates into lower production losses. The association between polymorphisms within genes encoding defensins – also belonging to AMPs – concerning milk production parameters was studied by Brodowska et al. [2019], and the study yielded results that can support appropriate animal selection for best performance, as well as an analysis by Ali et al. [2020] showed that another gene, *Jak2*, can be an essential candidate gene, and SNPs studied within it can be helpful to genetic markers for traits related to production and mastitis. Research by Rasheed et al. [2020] showed an association between SNPs located in the *CD4* gene promoter gene and dairy cattle production traits and mastitis indicators. Based on this, the *CD4* gene was also suggested as a potential candidate gene, and SNPs were suggested as molecular markers for mastitis resistance in dairy cattle.

Studies of the association between cathelicidins and milk performance parameters are relatively scarce, but due to the functions performed by these proteins, they are an interesting topic. A study conducted by Addis et al. [2017] revealed that the level of cathelicidins in milk increases due to pathogens in the mammary gland, making cathelicidins a good marker for determining mastitis. Additionally, a study by Wollowski et al. [2021]

shows that the level of cathelicidins in milk increases markedly during subclinical and clinical mastitis, while elevated protein values are observed only in milk collected from infected teat quarters. Studies by Zhang et al. [2015] have shown that cathelicidins are a promising biomarker of mastitis. Moreover, there is a correlation between the number of somatic cells in milk and the presence of cathelicidins, the concentration of which increases as the number of somatic cells increases, indicating their increased secretion in cows with ongoing subclinical and clinical mastitis. A study conducted on Gir cattle by Liao et al. [2013] showed that cathelicidins play an important antimicrobial role in this breed, and their potential can be used to improve animal husbandry. A study by Hiller et al. [2020] showed that for SNP 807 *G>A* within the *CATHL2* gene, the highest milk yield from cows, with the lowest number of somatic cells in milk, was obtained for the heterozygous genotype.

In our study, statistically significant differences were observed. For milk yield in cows, the highest value was observed for the *TT* genotype for the *CATHL2/Hha*I SNP and *GG* for the *CATHL3/Hin*fI SNP. The highest fat content was observed in cows with the *AA* and *AT* genotypes for the *CATHL2/Dde*I polymorphism and *GG* for the *CATHL2/Rsa*I polymorphism. The highest protein content in milk was in cows with the *AT* genotype for the *CATHL2/Dde*I polymorphism, *GG* for *CATHL3/Tsp*RI, *CC* for *CATHL3/Nla*III, and *TG* for *CATHL3/Hin*fI. The highest milk lactose content was in cows with the *CC* genotype for the *CATHL2/Hha*I SNP, *AA* for *CATHL2/Dde*I, *CC* for *CATHL2/Rsa*I, *TG* for *CATHL3/Tsp*RI, and *CC* for *Nla*III.

Notably, the results obtained for the number of somatic cells showed that the lowest number of somatic cells was observed for the heterozygote genotype for the *CATHL2/Hha*I SNP and the heterozygote genotype for *CATHL2/Rsa*I.

CONCLUSIONS

The study showed statistically significant differences between selected polymorphisms and milk performance parameters. Based on the results, it is possible to observe a tendency for higher milk yields with higher fat, protein, and lactose contents and lower somatic cell counts in heterozygous genotypes for *CATHL2/Hha*I and *CATHL2/Dde*I and *CATHL3/Nla*III polymorphisms, while for polymorphisms, *CATHL2/Rsa*I – *CC* genotype, *CATHL3/Tsp*RI – *GG* genotype and *CATHL3/Hin*fI – *GG* genotype were observed.

Based on the study, it was assessed that the results could be a prelude to expanding research in search of relationships between selected polymorphisms and parameters of dairy performance. In the future, they may provide a good direction in preparing selection programs in cattle breeding, however it is necessary to expand analysis on larger number of herds to confirm observed results and tendencies.

ACKNOWLEDGEMENT

The study was financed from funds allocated to maintenance of the research potential of the research units.

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IDENTYFIKACJA POLIMORFIZMÓW POJEDYNCZEGO NUKLEOTYDU W OBRĘBIE GENÓW KODUJĄCYCH BAKTENCYNĘ-5 I BAKTENCYNĘ-7 W POWIĄZANIU Z CECHAMI PRODUKCJI MLEKA

STRESZCZENIE

Baktencyny należą do białek z rodziny katelicydyn bydlęcych, a ich rola w organizmie jest istotnie ważna. Badania obejmowały analizę związku pomiędzy polimorfizmami w obrębie genów *CATHL2* i *CATHL3* kodujących białka: Bac-5 i Bac-7. Badaniami objęto stado krów rasy polskiej holsztyńsko-fryzyjskiej odmiany czarno-białej. Przeprowadzono badania z użyciem metody PCR-RFLP z wprowadzeniem modyfikacji ACRS. Na podstawie badań uzyskano wyniki, w których zaobserwowano, że różne warianty genetyczne w obrębie badanych polimorfizmów były istotnie związane z wybranymi parametrami użytkowości mlecznej, takimi jak: wydajność mleczna ($P \leq 0{,}01$), zawartość tłuszczu ($P \leq 0{,}01$), białka ($P \leq 0{,}01$ i $P \leq 0{,}05$) i laktozy ($P \leq 0{,}01$ i $P \leq 0{,}05$) oraz liczbę komórek somatycznych ($P \leq 0{,}01$ i $P \leq 0{,}05$).

Słowa kluczowe: *CATHL2*, *CATHL3*, bydło mleczne, SNP

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