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Analysis of the effect of polymorphisms within the *CATHL7* gene on dairy performance parameters

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Abstract

Introduction: Antimicrobial peptides, including cathelicidins, play a significant role in farm animals, influencing animal welfare, immunity, and thus the quality of animal products. **Material and Methods:** The study used amplification-created restriction site and PCR-restriction fragment length polymorphism to analyse single nucleotide polymorphisms of the *CATHL7* gene encoding the BMAP-34 protein in cattle, at positions 2,383 *G* > *C* and 2,468 *G* > *C*. The material was collected from 279 Polish Black-and-White Holstein-Friesian dairy cows. **Results:** There were statistically significant differences between milk performance parameters in cows with the *CATHL7/Hha*I and *CATHL7/Hin*fI genotypes. In the case of the *CATHL7/Hha*I polymorphism, the highest milk yield and protein and lactose content and the lowest somatic cell count in milk were observed for the *CC* genotype, while the fat content was the highest in milk from cows with the GG genotype. In the case of the *CATHL7*/*Hin*fI polymorphism, the highest protein and lactose content in milk was observed for the *CC* genotype. **Conclusion:** The results were statistically significant, which suggests that the search for relationships can be continued, and that the results can be used to improve selection programmes supporting dairy farming.

Keywords: ACRS, BMAP-34, cathelicidins, Holstein-Friesian cows, milk production traits.

Introduction

Cathelicidins are antimicrobial peptides (AMPs), which together with defensins form a large group of cationic peptides. They are part of the immune system of many vertebrates, including humans and farm animals. Cathelicidins are primarily found in cells of the immune system, particularly in macrophage granules. This peptide family is important in the host immune system because it takes part in the immune response against pathogens, including bacteria (Gram-positive and Gram-negative), viruses and fungi. Because of their antimicrobial activity, cathelicidins play an essential role in immunomodulation, *e.g.* by inhibiting apoptosis, stimulating cytokinesis, neutralising lipopolysaccharides, supporting wound healing, and regulating the host's immune response (32).

Cathelicidins are secreted in epidermal cells and glands of various organs – on the skin and in the gastrointestinal oral and nasal cavity, and udder lining epithelia. Secretion of these proteins in sweat, milk, and saliva has also been noted (24).

Genes encoding cathelicidins have been found in many animals, both invertebrates and vertebrates, but especially in mammals, in which more than 30 members of the cathelicidin family have been identified (18). One cathelicidin has been described in humans, while in cattle seven cathelicidin genes have been identified on chromosome 22 (36). The genes encoding proteins from the cathelicidin family are composed of four exons divided by three introns. The structure of each bovine cathelicidin is similar. It consists of three main elements: the N-terminal signal peptide, the cathelin domain, and the C-terminal variable region, which is responsible for the functions of cathelicidins, particularly their antibacterial, antiviral, antifungal, and immunomodulatory activity. The first three exons encode both the signal peptide and the cathelin domain, while the variable

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region is encoded by exon 4. The variable region's role validates investigating the potential influence of polymorphisms in exon 4 on selected performance traits in a group of animals (2).

Cathelicidins are increasingly attracting attention because of their functions in various animal species, including mammals, amphibians, and birds. Pig cathelicidin research by Ahn *et al.* (3) indicated the potential use of genetic testing in animal breeding. Chen *et al.* (8) described the characteristics of tiger frog cathelicidin, confirming a similar structure of the gene precursor to that of cathelicidin genes in other species, and at the same time observing a potential strong antibacterial effect against pathogenic microorganisms. Wang *et al.* (35) investigated avian cathelicidins found in chickens, pheasants, quails, turkeys and pigeons as potential therapeutic agents with antimicrobial properties that could replace antibiotics following more detailed research. In addition, the role of cathelicidins in the immune response has been observed in research in sheep (11).

In dairy cattle breeding, it is crucial to ensure the highest milk quality, to meet the expectations of consumers. Milk quality is influenced by both genetic and non-genetic factors, including nutrition, housing, hygiene and milking method (9). One of the most common problems in dairy cattle farming is mastitis, caused by numerous pathogenic bacteria. The infection causes a reduction in milk production, resulting in further economic losses in addition to those arising from the cost of veterinary treatment and from deaths in the herd. For this reason, it is essential to improve udder health by introducing tools for monitoring and prevention of infection (23).

Mastitis is one of the most commonly diagnosed diseases in dairy cattle. The most common aetiological agent of mastitis is bacteria of the *Staphylococcus* genus. The growing resistance of bacteria to antibiotics raises the importance of the search for alternative treatment and prevention methods. Innovative agents that could be used in antimicrobial therapy include cathelicidins, of which the potential use to treat udder infections has already been the subject of research (38). The growing antibiotic resistance of microorganisms pathogenic to farm animals is leading to the search for alternative prophylactic and therapeutic options, including among antimicrobial peptides (21).

Mastitis is manifested by an increase in the somatic cell count (SCC) in milk. A study by Smolenski *et al.* (31), in which the udder was directly infected with mastitis-causing bacteria, found an increase in the level of cathelicidins in milk during the initial phase of infection, which was associated with an increase in SCC. Studies to detect polymorphism within genes encoding proteins of the cathelicidin family are relatively scarce.

The search for dairy cattle genes of which variants influence animal performance has intensified over the years. Scientists are attempting to identify SNPs of various genes which can have a significant impact on the production levels of livestock, including dairy cows, focusing mainly on their potential effects on milk yield, protein and fat content and on milk SCC. In addition to the use of genetic tests to assess dairy performance, it is worth emphasising their potential use to improve the health of farm animals, and thus the quality of the products obtained from them (26).

Gillenwaters *et al.* (13) found 60 SNPs and five insertion-deletion mutations in four cathelicidin genes, *CATHL2*, *CATHL5*, *CATHL6*, and *CATHL7*, for ten cattle breeds. Molecular biology has a wide range of methods that can be used to improve breeding effects through the use of molecular markers associated with selected performance characteristics (19). These methods include gene and genomic selection, marker-assisted selection, and genomic assessment of breeding value, which successfully facilitate selection work and the creation of appropriate breeding programmes (25).

Marker-assisted selection is very useful due to its multi-faceted applications in breeding. It can be used to analyse traits that may be sex restricted, such as milk yield and performance traits, and can be a valuable tool supporting standard selection programmes with the potential to improve the genetic profile of the herd (34).

Essential techniques, such as the polymerase chain reaction (PCR) with its various modifications, are helpful tools enabling genotyping of animals in terms of selected genes and their potential impact on individual performance traits. The results can be used to implement marker-assisted selection. This makes it possible to perform a series of tests to search for and assess interesting genetic markers that could potentially be included in research aimed at improving the selection and breeding of animals (15).

Special attention should be focused on studies related to SCC, due to the cell count's potential role in the selection of cows showing increased resistance to pathogens or in the development of rapid tests for identification of mastitis, including subclinical forms, in milk. Wollowski *et al.* (37) conducted a study using milk from three groups of cows: healthy animals, those with symptoms of subclinical mastitis, and those with clinical mastitis. They measured the SCC and the presence and level of bovine cathelicidins in milk samples using an ELISA test. The results indicated differences in the presence of cathelicidins in the milk: in healthy cows the level of *CATHL* was practically undetectable, while the cathelicidin level was higher in cows with subclinical mastitis or clinical mastitis (37).

There is a need for further knowledge on the potential positive impact of cathelicidins on the condition, health, and production characteristics of animals. The study aimed to estimate the effect of polymorphisms occurring within the *CATHL7* gene encoding the BMAP-34 protein on selected parameters of the dairy performance of Polish Black-and-White Holstein-Friesian cows.

Material and Methods

The material consisted of 279 Polish Black-and-White Holstein-Friesian dairy cows, including 56 heifers, all in their second lactation. The animals were kept on a farm located in the Opolskie voivodeship. They were housed in a stand-alone system and fed using total mixed rations. They were milked twice a day using an automatic milking system and provided the investigated milk samples in the summer of 2022. Data on milk yields of cows were obtained from records of dairy performance evaluation by the Polish Federation of Cattle Breeders and Milk Producers. None of the cows suffered from clinical mastitis during the study period, while subclinical mastitis was detected in 7% of animals.

The SCC was log-transformed (LnSCC) according to Ali and Shook (4) and expressed as thousands/mL of milk, so that the conditions of normal distribution were met for this parameter as well as for daily milk yield (kg) and protein and fat content (%) in milk.

The first step in the analysis was DNA extraction. Peripheral blood was collected from the jugular vein of each cow. Sterile tubes containing the K3 ethylenediaminetetraacetic acid anticoagulant were used to preserve the sample. Isolation of DNA was achieved using the whole blood MasterPure DNA isolation kit (Epicenter Technologies, Mumbai, India) according to the manufacturer's recommendations.

The genotypes of individual animals were analysed by PCR-restriction fragment length polymorphism. Two polymorphic sites within exon 4 of the *CATHL7* gene were analysed. The SNP polymorphisms occur at positions 2,383 *G* > *C* (*rs448708946*) and 2,468 *G* > *C* (*rs477221357*). The fragment of the *CATHL7* gene was amplified by PCR using appropriate specific primer sequences, which were designed using a modification of the amplification-created restriction site (ACRS) method at position 2,381 *T* → *C*. The primer sequences were designed based on the sequence of the *CATHL7* gene (EU380715) and are as follows (the mismatched nucleotide is underlined): forward 5′-TCCTGGTTCACAGATTCAGAG<u>C</u>G-3′ and reverse 5′-CCTCACCCATCTGGGAGTTA-3′.

The PCR reaction conditions, product length, restriction enzymes used for digestion, genotypes, and length of the fragments after restriction enzyme cleavage are shown in Table 1.

The next stage of the analysis was digestion of the PCR products with restriction enzymes *Hha*I and *Hin*fI at 37°C for a minimum of 3 h. The PCR products and restriction fragments were assessed by electrophoresis on a 3% agarose gel with ethidium bromide and using the pUC19/*Msp*I marker. In addition, the results were visualised and archived using a kit for documentation and analysis of agarose gels (Figs 1 and 2).

Statistical analysis of the results included the relationship between different genotypes, different combinations of genotypes, and selected performance parameters, *i.e.* daily milk yield (kg), protein and fat content (%) in milk, and LnSCC.

Statistical analysis of the relationship between *CATHL7/Hha*I and *CATHL7/Hin*fI polymorphisms and milk performance parameters was performed using Statistica 12 software (StatSoft, now Tibco, Tulsa, OK, USA). Mean values ($\bar{x}$) and standard deviations (SD) were calculated, and one-way analysis of variance was performed using Duncan's multiple range test. The Kruskal–Wallis test was used to determine the significance of differences in the mean LnSCC. Hardy–Weinberg equilibrium was tested by calculating the chi-squared statistic (χ^2).

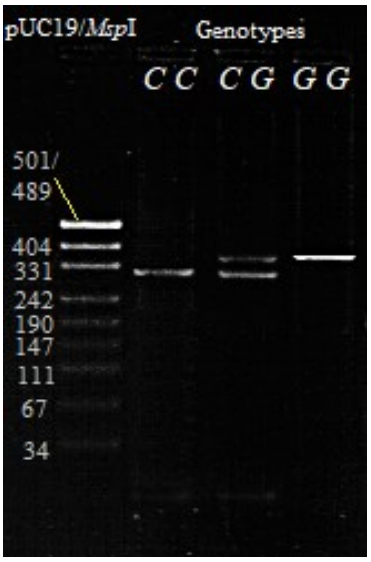


Fig. 1. Agarose gel electrophoresis (3%) of PCR-restriction fragment length polymorphism result for *CATHL7/Hha*I polymorphism. From left: pUC19/*Msp*I DNA ladder (501/489, 404, 331, 242, 190, 147, 111/110, 67 and 34 base pairs (bp)), *CC* genotype (324 and 23 bp), *CG* genotype (347, 324 and 23 bp), and *GG* genotype (347 bp); the 23 bp fragment is not visible because of its size and diffusion in gel

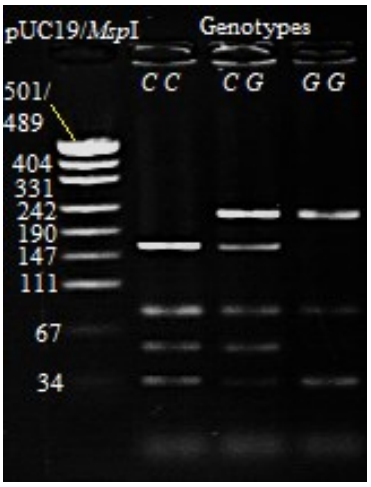


Fig. 2. Agarose gel electrophoresis (3%) of PCR-restriction fragment length polymorphism result for *CATHL7/Hin*fI polymorphism. From left: pUC19/*Msp*I DNA ladder (501/489, 404, 331, 242, 190, 147, 111/110, 67 and 34 base pairs (bp)), *CC* genotype (161, 81, 58, 34 and 14 bp), *CG* genotype (219, 161, 81, 58, 34 and 13 bp), and *GG* genotype (219, 81, 34 and 13 bp)

Table 1. PCR conditions, product size, restriction enzyme, and fragment size after restriction endonuclease digestion

SNP	PCR Conditions	Product size	Restriction enzyme	Genotype	Fragment size after digestion
2,383 *C > G*	initial: 95ºC/5 min 35 cycles: denaturation:95ºC/30 s annealing: 52ºC/45 s extension:72ºC/30 s final extension: 72ºC/7 min	347 bp	*Hha*I	*CC*	324, 23 bp
				CG	347, 324, 23 bp
				GG	347 bp
2,468 *C > G*			*Hin*fI	*CC*	161, 81, 58, 34, 14 bp
				CG	219, 161, 81, 58, 34, 13 bp
				GG	219, 81, 34, 13 bp

bp – base pairs

Table 2. Genotype and allele frequencies of *CATHL7*

	N	Genotype frequencies		Allele frequencies		χ^2
*CATHL7/Hha*I	169	*CC*	0.606	*C*	0.769	1.897
	91	*CG*	0.326	*G*	0.231	
	19	*GG*	0.068			
*CATHL7/Hin*fI	144	*CC*	0.516	*C*	0.722	0.208
	115	*CG*	0.412	*G*	0.278	
	20	*GG*	0.072			

N – number of cows

Table 3. Means and standard deviation of traits in relation to genotypes

N	Genotype	Milk yield kg	Fat content %	Protein content %	Lactose content %	LnSCC
			*CATHL7/Hha*I			
138	*CC*	$33.93^{A} \pm 7.86$	$3.84^{A} \pm 0.72$	$3.52^{A} \pm 0.33$	$4.95^{AB} \pm 0.16$	$4.18^{A} \pm 0.82$
68	*CG*	$33.81^{B} \pm 8.18$	$3.80^{B} \pm 0.75$	$3.52^{B} \pm 0.35$	$4.89^{B} \pm 0.17$	4.28 ± 0.84
17	*GG*	$30.42^{AB} \pm 6.29$	$4.07^{AB} \pm 0.70$	$3.59^{AB} \pm 0.37$	$4.90^{A} \pm 0.16$	$4.34^{A} \pm 0.75$
			*CATHL7/Hin*fI			
117	*CC*	33.48 ± 7.59	3.87 ± 0.71	$3.53^{A} \pm 0.34$	$4.94^{a} \pm 0.16$	4.18 ± 0.85
91	*CG*	33.87 ± 8.34	3.81 ± 0.77	$3.51^{a} \pm 0.34$	$4.91^{a} \pm 0.16$	4.27 ± 0.81
15	*GG*	34.42 ± 7.89	3.80 ± 0.67	$3.47^{Aa} \pm 0.31$	4.92 ± 0.17	4.25 ± 0.73

N – number of cows; LnSCC – natural log somatic cell count; [a] – values in a row with different letters differ significantly ($P \leq 0.05$); [A, B] – values in a row with different letters differ highly significantly ($P \leq 0.01$)

The following general linear model was used: $Yijkl = \mu + ai + bj + ck + dl + eijkl$

where $Yijkl$ is the value of a given trait in an individual, μ is the average value of the trait in the herd, ai is the fixed effect of the genotype ($i = 1, 2, 3$), bj is the fixed effect of the year of calving ($j = 1, 2, 3$), ck is the fixed effect of the month of calving ($k = 1, 2, 3, ..., 12$), dl is the random effect of the sire ($l = 1, 2, 3, ..., 13$), and $eijkl$ is the sampling error.

Results

Three genotypes were identified for *CATHL7/Hha*I (*CC*, *CG* and *GG*) and three genotypes for *CATHL7/Hinf*I (*CC*, *CG* and *GG*). The frequencies of individual genotypes and alleles are presented in Table 2. Hardy–Weinberg equilibrium was maintained in the population of cows.

The results obtained for the relationship between the genotypes of the polymorphisms and selected dairy performance parameters are presented in Table 3. In the case of the *CATHL7/Hha*I polymorphism, cows with the *CC* genotype were shown to have the highest daily milk yield, fat content, protein content, and lactose content, while cows with the *GG* genotype had the lowest values for these parameters. The differences were statistically confirmed as highly significant ($P \leq 0.01$). Statistically significant differences for the parameters were also found between the *CG* and *CC* genotypes ($P \leq 0.05$). Differences between the genotypes were observed for LnSCC, which was lowest in milk from cows with the *CC* genotype and highest in milk from those with the *GG* genotype. All differences were confirmed statistically as highly significant ($P \leq 0.01$).

In the case of the *CATHL7/Hin*fI polymorphism, protein content was highest in milk from cows with the *CC* genotype and lowest in milk from those with the *GG* genotype, and the difference was statistically highly significant ($P \leq 0.01$). The heterozygous genotype was associated with higher protein content than the *GG* genotype, and the difference was statistically significant ($P \leq 0.05$). Lactose content was highest in milk from cows with the *CC* genotype and lowest in those with the *CG* genotype. The difference was confirmed as statistically significant ($P \leq 0.05$). No statistically significant differences were shown for daily milk yield or fat content, but some trends were observed: daily milk yield was highest for cows with the *GG* genotype and lowest for those with the *CC* genotype, while fat content was highest in the

milk of cows with the *CC* genotype and lowest in milk from *GG* cows. In the case of SCC there were also no statistically significant differences between genotypes, but LnSCC was lowest in milk from cows with the *CC* genotype and highest in the milk of *CG* cows.

Discussion

Research to identify candidate genes associated with dairy performance parameters in cattle is carried out in order to improve animal welfare and selection programmes (29). The SCC in the milk of dairy animals is a good indicator of quality and udder health (30). An increase in SCC is a symptom of clinical or subclinical mastitis, which can cause significant economic losses and have a long-term impact on the health of the entire herd (12).

To increase the efficiency of breeding for dairy performance, researchers are exploring the potential uses of genetic testing for genes encoding proteins that may affect milk performance parameters (1). A study conducted on the *CATHL2* gene by Hiller *et al.* (16) found statistically significant differences in both dairy performance parameters and SSC. Research on the connection between genes encoding antimicrobial peptides and milk performance parameters has also been carried out using defensin genes. The relationship between polymorphisms within the defensin genes and milk performance parameters in cattle was first analysed by Ryniewicz *et al.* (27). The study showed that defensin genes could be used as genetic markers in breeding programmes selecting for individuals with increased resistance to mastitis. Moreover, research by Bagnicka *et al.* (6), Krzyżewski *et al.* (20), and Brodowska *et al.* (7) indicated the existence of a relationship between polymorphisms and milk yield in cattle.

The results of the present study suggest a relationship between the polymorphisms tested and parameters of milk performance. In the case of *CATHL7/Hha*I polymorphism, statistically significant results were obtained for the parameters tested, and it is worth noting that yield and protein content were highest for cows with the *CC* genotype, with high content of fat and lactose and the lowest SCC. In the case of the *CATHL7/Hin*fI polymorphism, the milk with the highest protein and lactose content and with the lowest (not confirmed statistically) SCC was obtained from cows with the *CC* genotype.

These polymorphisms are mapped in exon 4, which is responsible for encoding the variable region of the BMAP-34 protein. The *CATHL7/Hha*I polymorphism at 2,383 *C* > *G* changes the encoded amino acid from alanine to glycine, while the *CATHL7/Hin*fI polymorphism at 2,468 *C* > *G* changes the amino acid from isoleucine to methionine. Thus in these polymorphisms nucleotide substitutions cause amino acid changes which may affect the characteristics and functioning of the synthesised BMAP-34 protein.

Research on the potential use of individual SNPs as markers has been conducted using various cattle breeds to study various performance traits. Ateya *et al.* (5) analysed the effect of polymorphism of the genes encoding lactoferrin – an antimicrobial peptide – and demonstrated a relationship between the genotypes obtained and susceptibility to mastitis.

Khan *et al.* (17) investigated the effect of polymorphisms in the genes *JAK2* and *DGAT1* and showed them to be associated with milk fat content and characteristics associated with mastitis.

There are relatively few scientific reports on the potential link between cathelicidins and performance parameters. There is great interest in the development of tests for the rapid diagnosis of mastitis in cows by detecting cathelicidins in their milk, as the concentration of cathelicidins increases during the course of mastitis. BMAP proteins have been shown to have antimicrobial functions, *e.g.* by Valdez-Miramontes *et al.* (33), who demonstrated that BMAP-34 has a strong effect against *Staphylococcus aureus*, which is recognised as one of the main pathogens causing mastitis.

Identification of genes affecting resistance to mastitis has been of interest to researchers for many years. Griffin *et al.* (14) mapped the bovine chromosome 22q24 region, which contains genes associated with antimicrobial functions, as well as quantitative trait loci (QTLs) associated with SCC in milk. They identified *LTF* as a candidate gene in mastitis immunity and genes from the cathelicidin family as being involved in antimicrobial immune responses, thus confirming that this region is an interesting site for the search for genetic markers. The identification of QTLs affecting mastitis, SCC, udder characteristics, and milk yield in cows was studied by Lund *et al.* (22) in regions of bovine chromosomes 5, 6, 8, 13, 22, 23, 24 and 25. Quantitative trait loci affecting health, reproduction and body conformation in cattle were identified on chromosome 22 in a study by Cole *et al.* (10). Sahana *et al.* (28) conducted a study to identify QTLs associated with clinical mastitis in dairy cattle in regions of bovine chromosomes 6, 13, 16, 19, and 20.

The characteristics of the proteins encoded by cathelicidin family genes make them potential targets in the search for genetic markers. There are studies of the relationships between polymorphisms within these proteins and studies of various parameters and relationships, focusing mainly on the relationship between the host immune system and cattle performance, including the influence of polymorphisms on meat performance parameters.

The search for genetic markers for use in livestock breeding programmes is a priority task for researchers. Cathelicidins are proteins performing numerous functions in the body and may be genetic markers which lend themselves to use in such programmes. Due to their antimicrobial activity, it is essential to learn more about their potential effects. Particularly noteworthy are

potential relationships between polymorphisms within the *CATHL7* gene and somatic cell count.

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