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Association between the polymorphism of bovine β4-defensin gene and milk traits in Holstein-Friesian cows as computed for standard (305 days) and the whole lactation*

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Associations were studied between the polymorphic forms of bovine β4-defensin gene and milk traits in cows during the standard (305 days) and the whole lactation. The study was carried out in the years 2004-2007 on 207 Holstein-Friesian cows yielding on the average 9600 kg milk/year. The animals were in their first to fifth lactation, maintained in loose barn and fed *ad libitum* with TMR (total mixed ration) composed of maize silage, wilted grass silage and concentrates supplemented with minerals and vitamins. The diets were formulated according to the INRA standards. Milk production data were collected from the individual cows' records during their consecutive lactations. The RFLP-*Nla*III method was used to identify the polymorphic forms of β4-defensin gene (*C* and *T*). A total of 616 records of standard and 837 of the whole lactation for milk, milk fat and milk protein yield and fat and protein content of milk were statistically evaluated using one-trait repeatability animal model with DMU package.

The cows of *CC* genotype yielded more milk than cows of genotype *CT* during both the standard (+181 kg, P≤0.05) and the whole lactation (+241 kg, P≤0.05). The *CT* cows produced milk with higher protein content (P≤0.01 and P≤0.05 for standard and whole lactation, respectively), but the protein yield did not differ significantly between the two genotypes in question. Moreover, no significant differences were identified in fat content of milk between the *CC* and *CT* genotype, but the fat yield was higher (P≤0.05) in homozygous *CC* cows.

KEY WORDS: cattle / defensin / gene polymorphism / milk traits / lactation

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Antimicrobial peptides (AMPs) comprise an important component of the innate immune system in protecting the host from microorganisms [Brahmachary *et al*. 2006]. A major family of AMPs found in plants, insects and mammals are defensins [Suresh and Verma 2006]. Defensis are small (29-49 amino acids and 3.5-6 kDa of molecular weight), cysteine-rich peptides whose structure is stabilized by three disulphide bounds, formed by six strongly conserved cysteines [De Smet and Contreras 2005]. The mammals' defensins are divided into three classes, the α-, β- and θ-defensins, based on different spacings of the six cysteine residues and arrangement of the disulfide bonds [Circo *et al.* 2002], but the θ-defensins were found only in plants and some primates, excluding humans [Lehrer and Ganz 2002]. All α- and most β-defensin genes occur in a cluster at the chromosome 8p23.1 of human genome, which is syntenic to 27th bovine chromosome [O'Brien *et al.* 1993]. Defensins exhibit a broad spectrum of activity against Gram-positive and Gram-negative bacteria, fungal species, and viruses [Jurevic *et al.* 2003]. In addition to killing invading microbes, these peptides have several other functions [Aarbiou *et al.* 2006]. The α-defensins are abundantly expressed in cells of the immune system, like Paneth cells and neutrophils and are also present in certain epithelia [Hollox *et al.* 2003, Vankeerberghen *et al.* 2005]. The expression of β-defensins is restricted mainly to epithelial cells that line the body and also to neutrophils and macrophages and can be induced by microorganisms and inflammatory factors [Schneider *et al.* 2005]. Their function in the immune system is dual. First, due to their positive charge, they can interact with and integrate into the membrane of pathogens, including bacteria, fungi, and enveloped viruses, to kill them. Second, they can attract cells of the acquired immune system and they play an important role in linking innate and adaptive immunity acting as "mini-chemokines" [Braida *et al.* 2004, Vankeerberghen *et al.* 2005]. Defensins are perhaps the most important family of antimicrobial peptides in humans and animals, and are also known for their antiviral activities. Sixteen bovine β-defensins are known, which expression was confirmed in leukocytes and in many tissues, including udder epithelial cells and somatic cells in milk [Ryan *et al.* 1998, Tarver *et al*. 1998, Diamond *et al*. 2000, Goldammer *et al.* 2004, Roosen *et al.* 2004, Bagnicka *et al.* 2006].

Our earlier study showed that polymorphism of bovine β4-defensin gene could be the genetic marker for the somatic cell count (SCC), fat, protein and lactose content of milk measured in milk samples taken during monthly control milking [Bagnicka *et al.* 2007].

The aim of the present study was to estimate associations between *Nla*III polymorphism of β4-defensin gene at 2239 nucleotide (GenBank accession no. AF008307) and milk, fat and protein yield, and fat and protein content of milk both during the 305-days and the whole lactation.

Material and methods

Animals

Used were records from the years 2004-2007 of 207 high-yielding dairy Holstein-Friesian (HF) cows. The average milk yield was about 9600 kg/year, containing 4.30% of fat and 3.43% of total protein. The cows remained between their first and fifth lactation and were kept in a loose barn at the Experimental Farm of the Polish Academy of Sciences, Jastrzębiec. The total mixed ratio (TMR) was applied, based on maize silage, wilted grass silage and concentrates supplemented with minerals and vitamins. The diets were formulated according to the INRA (National Institute for Agricultural Research, France) standards. Twice a day the cows were machine-milked. Milk production data were withdrawn from the records kept for every cow, following three consecutive lactations.

The Local Ethics Commission, Permission No. 17/2005, approved all procedures carried out with all the animals.

DNA isolation from whole blood

Blood was collected on K_2-EDTA and stored at -25°C for a few weeks or at -75°C up to several months. The isolation of DNA from whole blood was done using a rapid method described by Kanai *et al.* [1994].

Identification of defensin genotypes

For the restriction fragment length polymorphism (RFLP) of β4-defensin gene the 393 bp-long fragment was PCR-amplified using the following primers [Bagnicka *et al*. 2007]:

forward: 5'-TGGCAGGAAGGAGGATGTAG-3' and

reverse: 5'-ACGGCACAAGAACGGAATAC-3'.

The forward primer covered a fragment of intron between nucleotides (nt) 2099 and 2119 (GenBank, accession no. AF008307), while the reverse primer matched a fragment of exon 2, between nt 2473 and 2492. The PCR product was digested with the *Nla*III (*Hin*III) endonuclease allowing an identification of the C2239T transition, and the restriction DNA fragments were analysed electrophoretically in 2% agarose gels. The amplified 393-bp amplicon is cut by *Nla*III endonuclease into 253 and 140-bp fragments (in the case of allele *T*). The identity of the analysed fragment of the β4-defensin gene was confirmed by sequencing the PCR products representing *CC*, *CT* and *TT* genotypes.

Milk analyses

Milk samples were taken from each cow once a month, in the course of routine milk recording, during the whole lactation. The samples were analysed by an independent laboratory and the results obtained were finally withdrawn from the Official Recording Milk Performance records. The data set contained the information about the milk, fat

and total protein yields and also mean fat and mean total protein content during the standard (305 days) and the whole lactation.

Statistical

A total of 616 records concerning the standard and 837 records of the whole lactation were withdrawn for 207 cows. In order to determine the relationship between the polymorphism of the β4-defensin gene and the investigated milk production traits, one-trait repeatability animal model with DMU package [Madsen and Jensen 2000] was used. The significance of differences between estimates of β4-defensin genotype effects was identified using Student's t-test. The model included the animal defensin genotype and parity as fixed effects and the animal additive genetic effect and permanent environmental effect of individual cow, as well as year-season of calving as random effects. According to their parity, the animals were divided into three classes with the third one covering parities higher than the second. The cows with lactations shorter than 270 days were excluded from statistical analysis. Also the animals carrying the *TT* genotype were not included in the statistical evaluation because of the small number of observations – the frequency of the *TT* genotype as reported by Bagnicka *et al*. [2007] was only 0.02.

Results and discussion

Estimates (means) and their standard deviations for both genotypes of the investigated traits as affected by β4-defensin genotype in standard lactations are shown in Table 1. The cows of *CC* genotype produced about 181 kg more milk than those of the genotype *CT* (P≤0.05). The milk of the latter contained more protein (P≤0.01) than that of *CC* cows. In spite of the higher total protein content of milk in the *CT* cows the total protein yield was the same in both groups – *CT* and *CC* – because of higher milk yield of the *CC* cows. The fat yield was higher in *CC* homozygotes (P≤0.05), although the fat content was similar in both genotype groups.

Table 1. Estimates (means) and their standard errors (SE) for milk, fat and total protein yield, and fat and total protein content of milk as related to β4-defensin genotypes in cows, computed for standard (305 days) lactation

Genotype	Number of records	Yield (kg)						Content (%)			
		milk		fat		total protein		fat		total protein	
		mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
CC	461	10044^{a}	350	395.5^{a}	13.1	322.3	11.1	4.05	0.08	3.37^{A}	0.03
CT	155	9863^{a}	378	387.2^{a}	14.0	319.4	12.1	4.04	0.09	3.41^{A}	0.03

^{aA}Within traits (columns) means bearing the same superscript differ significantly at: small letters – P≤0.05; capitals – P≤0.01.

Similar associations were found between polymorphic forms of β4-defensin gene and milk traits measured in the whole lactation (Tab. 2). In that case also the milk yield was found higher in *CC* homozygotes than in heterozygotes (P≤0.05). The higher milk yield in the *CC* group of cows resulted from their longer lactation and can also be attributed to the β4-defensin genotype. Cows of the *CC* genotype produced more milk (by about 241 kg) and their lactation was by 8 days longer than in those of genotype *CT*. However, cows of the *CT* genotype showed higher total protein content of milk (P≤0.05).

Our earlier study indicated the lack of relationship between the β4-defensin gene polymorphism and milk yield as measured using milk test-day model [Bagnicka *et al.* 2007] while the results of the current report indicate the higher milk yield in cows of the *CC* genotype in the standard as well as in the whole lactation. Moreover, the contrasting results were obtained for the fat content. The present report reveals no differences between genotypes in the mean fat content estimated during the whole as well as standard lactation, while in the earlier study the difference in fat content evaluated with the milk test-day model [Bagnicka *et al*. 2007] appeared significant. In the earlier study the milk of *CC* homozygotes contained more fat than that of heterozygotes (P≤0.01). Those discrepancies are difficult to explain. In both studies the total protein content of milk was higher in cows carrying the *CT* genotype (P≤0.01 and P≤0.05 for standard and whole lactation, respectively, and P≤0.05 for test-day model).

There is only a limited information available [Bagnicka *et al.* 2007] on the relationship between polymorphic forms of β4-defensin gene and milk traits in dairy cows. Existing of such associations was confirmed by the present report. Earlier, only associations between combined defensin genotypes and milk production traits were investigated [Ryniewicz *et al*. 2002, Ryniewicz *et al*. 2003, Wojdak-Maksymiec *et al*. 2006]. In the currently investigated population of HF cows we found only a few animals of the *TT* β4-defensin genotype.

Table 2. Estimates (means) and their standard errors (SE) for milk, fat and total protein yield, and fat and total protein content of milk as related to β4-defensin genotypes in cows, computed for the whole lactation

Genotype	Number of records	Lactation days	Yield (kg)						Content (%)			
			milk		fat		total protein		fat		total protein	
			estimate	SE	estimate	SE	estimate	SE	estimate	SE	estimate	SE
CC	612	340[a]	10185[a]	458	400.0[a]	17.5	345.7	11.3	4.15	0.09	3.22[a]	0.03
CT	225	332[a]	9945[a]	481	391.3[a]	18.2	342.7	12.0	4.13	0.10	3.25[a]	0.03

[a]Within traits (columns) means bearing the same superscript differ significantly at P≤0.05.

We suppose that cows carrying *T* allele were eliminated from the herd because of their low productivity. This hypothesis is confirmed by the lower milk yield of heterozygotic *CT* cows, *i.e*. those carrying the allele *T*. The relations presented here and concerning the total protein content of milk are in accordance with our earlier results obtained on the other population of HF cows and using the milk test-day model to establish relationships between the polymorphic forms of β4-defensin gene (*CC* and *CT*) and milk traits [Bagnicka *et al*. 2007].

Recently, many investigations have been conducted to detect the polymorphisms of other genes as markers for production traits in cattle. They referred to bovine growth hormone [Pawar *et al.* 2007], bovine STAT5A [Brym *et al.* 2004], IGF1 [Siadkowska *et al.* 2006], leptin [Liefers *et al.* 2002], β-lactoglobulin [Kuss *et al.* 2003], casein genes [Braunschweig *et al.* 1999] and others. Based on our present and earlier results one can conclude that also bovine β4-defensin gene could be used as a marker in selecting dairy cattle to improve milk traits, simultaneously with increased resistance to infection of the mammary gland. However, further studies should be carried out to confirm these relationships, and before the bovine β4-defensin gene would be used as a marker of milk traits in mass selection of dairy cows.

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Zależność między polimorfizmem genu bydlęcej defensyny β4 a cechami mleczności krów hf w okresie 305-dniowej i pełnej laktacji

S t r e s z c z e n i e

Poszukiwano zależności między formami polimorficznymi genów bydlęcej β-defensyny 4 a cechami mleczności krów w okresie 305-dniowej (standard) i pełnej laktacji dla ewentualnego wykorzystania wyników w selekcji bydła mlecznego. Badania przeprowadzono na danych uzyskanych z kontroli użytkowości 207 krów rasy hf (przeciętna roczna wydajność 9600 kg mleka) w latach 2004-2007. Krowy znajdowały się w 1-5 laktacji, były utrzymywane w oborze wolnostanowiskowej oraz żywione do woli według systemu TMR dawkami złożonymi z kiszonki z kukurydzy, kiszonki z przewiędniętych traw oraz mieszanki pasz treściwych uzupełnianymi mieszanką mineralno-witaminową. Dawki bilansowano według INRA. Próbki mleka pobierano od poszczególnych krów w okresie całej laktacji. Genotypy β-defensyny 4 (*C* i *T*) oznaczano metodą RFLP-*Nla*III. Do analizy statystycznej wykorzystano 837 rekordów dotyczących pełnej laktacji oraz 616 rekordów dotyczących laktacji 305-dniowej. W analizie uwzględniono wydajność mleka, tłuszczu i białka oraz procent tłuszczu i białka z wykorzystaniem jednocechowego, powtarzalnościowego modelu zwierzęcego, w pakiecie DMU. Krowy o genotypie *CC* produkowały istotnie więcej mleka niż krowy o genotypie *CT*, zarówno w okresie laktacji 305-dniowej, jak pełnej (odpowiednio +181 kg i +241 kg). Mleko krów o genotypie *CT* zawierało istotnie więcej białka niż mleko krów o genotypie *CC*, jednak między omawianymi genotypami nie udowodniono istotnych różnic w wydajności tego składnika. Nie stwierdzono także różnic między genotypami w zawartości tłuszczu w mleku. Wydajność tłuszczu była istotnie wyższa w grupie krów o genotypie *CC*.