

ASSOCIATION OF KAPPA CASEIN POLYMORPHISM WITH MILK YIELD AND MILK PROTEIN GENOMIC VALUES IN COWS REARED IN LITHUANIA

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Summary. Aim of the study was to investigate polymorphism of bovine kappa casein gene in Lithuanian milk cattle population and to evaluate relation of certain genotypes with milk production traits expressed by genome scores. DNA samples were collected from 189 milk breed cattle reared in Lithuania. DNA was extracted from blood by salt method (Miller et al., 1998). Bovine kappa casein gene polymorphism study was performed by PCR-RFLP method (Soria et al., 2003). After PCR 935 bp fragment was received, which was digested with restriction enzymes HaeIII / HindIII and the fragments were separated in the 3% agarose gel, what allowed to identify the kappa casein A, B, E alleles. A allele with frequency 0.72, E allele with frequency 0.05. B allele, which can be used to carry out selection to improve milk processing properties was found 0.23 frequency. Six genotypes were identified: AA, AB, AE, BB, BE, EE at different frequencies. The most common was the AA genotype, which had 49.2 % of the tested animals. EE was least frequently occurring genotype, which had only 0.5% of the tested animals. The biggest influence on the milk processing properties having BB genotype was found in 2.1% of the cows. Cows with kappa casein AA genotype had the highest genomic scores for milk yield (6.05), observing statistically significant difference between kappa casein AA and AB genotypes ($P < 0.05$); the highest protein amount genomic values had kappa casein BB genotype cows (6.25), the highest protein percent genomic scores had BB and BE genotype cows. Dispersion analysis showed that 2.8 % of milk yield variation expressed by genomic scores, 0.2% of protein amount variation and 3.3 % of protein percent variation ($P < 0.05$) are dependent from kappa casein gene genotype. These results create opportunities to improve the properties of milk processing in Lithuanian dairy cattle population.

Keywords: kappa casein, polymorphism, cattle, PCR-RFLP

Background. Animal productivity, milk amount, milk composition, milk protein and fat amount, and composition, milk suitability for manufacturing and other farm traits are inherited from generation to generation. Farm traits expression and functional properties are dependent on genes that animals have. Cow milk quality and quantity are also dependent on feeding, milking, keeping conditions and many other factors but even when all the factors are optimally balanced, the part of productivity, dependent on heritability, stays unchanged.

The milk proteins are the most important components of milk in human nutrition. Today the dairy industry has the technological possibilities to produce much more different kinds of milk products. The manufacturing properties of milk are related to the composition of proteins in milk (Lunden et al., 1997). All of casein proteins are the major constituencies (80%) of total proteins in cattle milk. (Grosclaude, 1988).

Milk casein proteins are coded by four genes Casein S1, Casein S2, Beta casein and Kappa casein found on chromosome 6 in 6q31 position (Threadgill and Womack, 1990). Total kappa casein gene length is 13 kb, composed from 5 egzones (Martin et al., 2002), but most sequences coding proteins are found in fourth egzone (Azevedo et al., 2008). Kappa casein is a one strand polypeptide, comprised from 169 amino acids with molecular weight 19,2 KDa. Kappa casein is only fraction of caseins, which contains S-amino acid and is not precipitated by calcium ions. In 1984 Eigel and co-authors published an article about the two variants of κ -casein, A and B, that differ in their amino acids at the positions 136 and 148. In position 136, Thr (ACC) is substituted by Ile (ATC), while in

position 148 there is Ala (GCT) instead of Asp (GTA) (Tinaev, 2003). The positions of these two point mutations are close to the glycosylation sites, which affects the protein structure as well as the glycosylation itself (Fox, 1992). Thus, these genetic variants are related to the milk properties of the dairy cows, milk instability during the milk processing as its suitability for cheese production. In recent years, the variations in the promoter region of the casein gene, and their influence for the expression of the milk proteins came into the scientists attention. In addition, changes in the flanking region, together or separately with the changes in the coding region, may affect the yield and quality of milk (Caroli et al., 2009; Hoogendoorn et al., 2003; Martin et al., 2002; Pauciuolo et al., 2013).

Kappa casein genetic variants are found in different frequencies in various milk cattle populations. Most European cattle breeds have very high frequency of kappa casein A allele (Jakob, 1992; Van Eenennaam and Medrano, 1991) and this allele is predominant in the Holstein cattle (Lin et al., 1986). For the selection it is very important to identify animals with B allele and BB genotype of kappa casein to increase the frequency of this allele in dairy cattle population. So our aim was to test kappa casein polymorphism in milk cattle reared in Lithuania, identify animals with favourable B allele and BB genotype and evaluate relation of certain genotypes with milk production traits expressed by genome scores.

Materials and methods

One hundred eighty nine milk breed cows reared in Lithuania were sampled at random. Blood samples were taken from jugular vein into EDTA containing tubes,

transported to laboratory and stored at -20°C until genomic DNA extraction. DNA was extracted by chloroform salt method (Miller et al., 1998). Genomic DNA content and purity were determined by spectrophotometric methods (DNA / RNA Reader, Pharmacia). Genomic DNA was stored at 4°C until

analysis. Statistical analysis performed by SPSS program.

Genotyping for kappa casein was done using PCR-RFLP method (Soria et al., 2003). The primers, PCR profile, PCR product size and restriction enzymes used for identification of milk protein are shown in Table 1.

Table 1. Primer, PCR profile, PCR product size and restriction enzymes used for identification of milk protein kappa casein polymorphisms

Milk protein	Primer	PCR profile			PCR product size	Restriction enzyme
		94 °C	3 min	30 cycles		
Kappa casein	CSN3 1 5'-AGCGCTGTGAGAAAGATG-3' CSN3 2 5'-GTGCAACAACACTGGTAT-3'	94 °C	30 s	30 cycles	935 bp	<i>HindIII</i>
		58 °C	30 s			
		72 °C	30 s			
		72 °C	5 min			<i>HaeIII</i>

The PCR reaction was carried out in GeneAmp PCR System 2700 (AppliedBiosystem). PCR mix – 12 μl ddH₂O; 5 μl 10xPCR buffer; 2.5 μl dNTP (2 mM); 3 μl MgCl₂ (50 mM); 2.5 μl CSN3 1 (20 pmol); 2.5 μl CSN3 2 (20 pmol); 0.5 μl BSA; 2 μl Taq (1U/ μl) polymerase (MBI Fermentas, Lithuania). The digestion of PCR product was performed using endonucleases *HaeIII*/*HindIII* (MBI Fermentas, Lithuania). 20 μl PCR product was digested with 10.5 μl restriction mix (8 μl ddH₂O, 2 μl 10xMbuf., 0.5 μl *HaeIII*/*HindIII*) (to separate alleles A, B, E). The samples were left in thermostate for night (15 h) at 37°C (table 2).

Table 2. PCR fragment sizes in bp of Kappa casein gene after the digestion with restriction endonucleases

Genotype	Restriction endonucleases, fragment sizes in bp	
	<i>HindIII</i>	<i>HaeIII</i>
A/A	935	641 + 294
A/B	935 + 520 + 415	641 + 294
A/E	935	641 + 496 + 294 + 145
B/B	520 + 415	641 + 294
B/E	935 + 520 + 415	641 + 496 + 294 + 145
E/E	935	496 + 294 + 145

Kappa casein genotypes were determined using 3 % agarose gel by electrophoresis 100 V 50 min. stained with ethidium bromide 15–20 min. EtBr was added to gels to visualize the analysis results under UV light in „MiniBisPro“ system (Herolab).

Genome analysis was performed by Igenity SNP panel identifying the genetic potential for dairy cattle traits - milk yield and protein amount and protein %. Different traits potentially related to milk yield, protein amount and protein % SNP's were tested. The IGENITY dairy cattle profile calculates scores for traits using multiple DNA markers. The largest score indicates the best genomic value for milk yield and milk amount and milk protein %. Combined results provide more complete picture of an animal's production potential. Genotypes

were rated in Igenity dairy cattle reference group to identify genomic scores relating to productive traits.

Results

The polymorphism of kappa-casein gene was investigated by PCR-RFLP method in 189 dairy cattle. Restriction of the obtained 935 bp PCR reaction products by *HinfI* and *HaeIII* restriction enzymes allowed determination of the frequencies of A, B and E alleles of kappa-casein in the investigated group of cattle.

Table 3. Kappa casein allele frequencies in Lithuanian milk cattle

Identified kappa casein alleles	Observed frequencies
A	0.721
B	0.227
E	0.052

Table 4. Kappa casein genotype frequencies in Lithuanian milk cattle

Kappa casein genotypes	Observed frequencies	Expected frequencies
AA	0.492	0.518
AB	0.386	0.327
AE	0.069	0.076
BB	0.021	0.052
BE	0.026	0.024
EE	0.005	0.003

The frequency of A allele was found to be highest – 0.721, while the lowest was of E allele - 0,052. B allele, which is important for the processing properties of milk, was found in 22.7% of cows investigated. A allele was more than three times more frequent than B allele and even 13.6 times more frequent than E allele (table 3).

The expected and predicted genotype frequencies did not differ statistically significantly ($\chi^2=6.255$; $P=0.282$).

Six different genotypes were identified in the group of investigated cattle: AA (0.492), AB (0.386), AE (0.069),

BB (0.021), BE (0.027), EE (0.005). Three homozygous genotypes AA, BB and EE were found and three heterozygous - AB, AE and BE. Out of the identified genotypes, the AA was found to be the most frequent. It was found in 49.2% of investigated cows. EE genotype

was found to be the least frequent. It was found only in 0.5% of investigated cattle. BB genotype, which highly influences the processing properties of milk, was found in 2.1% of cows (table 4).

Table 5. Distribution of Kappa casein genotypes according milk yield genomic scores

Kappa casein genotypes	Milk yield genomic scores									
	1	2	3	4	5	6	7	8	9	10
AA			1.1%	12.6%	16.1%	34.5%	24.1%	9.2%	2.3%	
AB		4.6%	9.2%	13.8%	16.9%	27.7%	15.4%	7.7%	4.6%	
AE			8.3%	8.3%	16.7%	25.0%	25.0%	16.7%		
BB				25.0%	25.0%	25.0%		25.0%		
BE				25.0%	25.0%		50.0%			
EE					100.0%					

Comparing different kappa casein genotypes according to milk yield genomic scores in kappa casein, AA genotype cow milk yield genomic scores varied from 3 to 9, most cows (34.5%) got 6 milk yield genomic scores; kappa casein AB genotype cow milk yield genomic scores varied from 2 to 9, most cows (27.7%) got 6 milk yield genomic scores; kappa casein AE genotype cow milk yield genomic scores varied from 3 to 8, same amount of cows (25.0%) got 6 and 7 milk yield genomic scores; in kappa casein BB genotype cow milk yield genomic scores varied from 4 to 8, same amount of cows (25.0%) got 4,5, 6 and 8 milk yield genomic scores; in kappa casein BE genotype cow milk yield genomic scores varied from 4 to 7, same amount of cows (25.0%) got 4 and 5 milk yield genomic scores; rest 50 % of cows got 7 milk yield genomic scores; all kappa casein EE genotype cows got 5 milk yield genomic scores. Pearson Chi-Square test shows no statistically significant influence of kappa casein gene to milk amount expressed by genomic scores ($P=0.838$) (table 5).

Comparing different kappa casein genotypes according to protein amount genomic scores in kappa casein, AA genotype cows protein amount genomic scores varied from 2 to 10, the most of cows (20.7%) got 6 and 8 protein amount genomic scores equally; kappa casein AB genotype cows protein amount genomic scores varied from 1 to 10, most cows (19.7%) got 7 protein amount genomic scores; kappa casein AE genotype cows protein amount genomic scores varied from 4 to 8, same amount of cows (33.3 %) got 6 and 7 protein amount genomic scores; in kappa casein BB genotype cows protein amount genomic scores varied from 4 to 9, all cows (25.0%) got same protein amount genomic scores; in kappa casein BE genotype cows protein amount genomic scores varied from 2 to 9, most cows (50.0%) got 9 protein amount genomic scores; all kappa casein EE genotype cows got 6 protein amount genomic scores. Pearson Chi-Square test shows no statistically significant influence of kappa casein gene to milk amount expressed by genomic scores ($P= 0.636$) (table 6).

Table 6. Distribution of Kappa casein genotypes according protein amount genomic scores

Kappa casein genotype	Protein amount genomic scores									
	1	2	3	4	5	6	7	8	9	10
AA		3.4%	2.3%	14.9%	16.1%	20.7%	12.6%	20.7%	6.9%	0.4%
AB	1.5%	6.1%	6.1%	9.1%	13.6%	18.2%	19.7%	16.7%	6.1%	0.7%
AE				25.0%		33.3%	33.3%	8.3%		
BB				25.0%	25.0%		25.0%		25.0%	
BE		25.0%		25.0%					50.0%	
EE						100.0%				

Comparing different kappa casein genotypes according to protein percent genomic scores in kappa casein, AA genotype cows protein percent genomic scores varied from 5 to 10, most cows (33.3%) got 7 protein percent genomic scores; kappa casein AB genotype cow milk protein percent genomic scores varied from 5 to 10, most cows (27.3%) got 7 protein percent genomic scores; kappa casein AE genotype cow milk protein percent genomic scores varied from 5 to 10, same amount of cows (25.0%) got 6 and 7 protein percent genomic scores; in kappa casein BB genotype cows milk protein percent

genomic scores varied from 6 to 10, same amount of cows (25.0%) got 9 and 10 protein percent genomic scores and the rest of cows (50.0 %) got protein 6 percent genomic scores; in kappa casein BE genotype cows protein percent genomic scores varied from 7 to 8, the most of cows (75.0%) got 8 protein percent genomic scores; all kappa casein EE genotype cows got 9 protein percent genomic scores. Pearson Chi-Square test shows no statistically significant influence of kappa casein gene to milk amount expressed by genomic scores ($P=0.2300$) (table 7).

Table 7. Distribution of Kappa casein genotypes according protein percent genomic scores

Kappa casein genotypes	Protein percent genomic scores									
	1	2	3	4	5	6	7	8	9	10
AA					8.0%	18.4%	33.3%	28.7%	10.3%	1.3%
AB					3.0%	21.2%	27.3%	21.2%	21.2%	6.1%
AE					8.3%	25.0%	25.0%	16.7%	16.7%	8.3%
BB						50.0%			25.0%	25.0%
BE							25.0%	75.0%		
EE									100.0%	

Table 8. Relation of Kappa casein genotypes to milk production traits genomic scores

Kappa casein genotypes	n	Milk amount genomic scores	Protein amount genomic scores	Protein percent genomic scores
AA	87	6.05±0,136 a	6.17±0,200	7.18±0,122
AB	65	5.54±0,215 b	6.02±0,257	7.55±0,159
AE	12	6.00±0,444	6.00±0,389	7.33±0,432
BB	4	5.75±0,854	6.25±1,109	7.75±1,031
BE	4	5.75±0,750	6.00±1,780	7.75±0,250
EE	1	5.00	6.00	9.00

a, b – averages marked in different letters in the column differ significantly (P <0.05)

Cows with kappa casein AA genotype had the highest genomic scores for milk yield (6.05), observing statistically significant difference between kappa casein AA and AB genotypes (P <0.05); the highest protein amount genomic values had kappa casein BB genotype cows (6.25), highest protein percent genomic scores had BB and BE genotype cows. EE genotype cow had genomic score for protein percent 9, but as it was only 1 cow, it can be a casual case (table 8).

Dispersion analysis showed that 2.8 % of milk yield variation expressed by genomic scores (P >0.05), 0.2% of protein amount variation (P >0.05) and 3.3 % of protein percent variation (P <0.05) are dependent from kappa casein gene genotype.

Discussion. For the first time association of Igenity dairy cattle genomic values for production traits with milk protein genotypes were tested. Cows with kappa casein AA genotype had the highest genomic scores for milk yield, observing statistically significant differences between kappa casein AA and AB genotypes; kappa casein BB genotype cows had highest genomic values for protein amount and protein percent, BE genotype cows - for protein percent. The IGENITY profile calculates scores for milk yield, fat amount, fat percent, protein amount and protein percent using multiple DNA markers. These markers identify genetic variations that help to regulate milk yield, protein and fat content, without decreasing fertility. The combined results provide more complete picture of an animal's production potential. The use of genomic information in genetic evaluation has brought revolutionary change in dairy. Genomic selection offers many advantages with regard to improve the rate of genetic gain in dairy cattle breeding programs. The most important factors that contribute to faster genetic gain include: a greater accuracy of predicted genetic merit for young animals; a shorter generation interval because of heavier use of young, genetically superior males and

females; an increased intensity of selection, because breeders can use genomic testing to screen a larger group of potentially elite animals. By increasing the accuracy and intensity of selection and shortening the generation interval, the rate of genetic progress for economically important dairy traits can be approximately doubled (Schaeffer, 2006; Pryce and Daetwyler, 2012).

Milk is very important in human diet. Human consume raw milk and different milk products such as cheese, curd, yogurt, ect. so milk manufacturing properties are very important. One of the modern ways to improve milk quality is genetic or biotechnological method such as MAS (Marker Assisted Selection) (Rachagani and Gupta, 2008; Dogru and Ozdemir, 2009). Caseins are one of the most important milk proteins. There are 14 alleles identified of the kappa-casein (A, A1, B, B2, C, D, E, F1, F2, G1, G2, H, I, J) (Caroli et al., 2009), but the A and B alleles are the most prominent, as were also found in our study (Chessa et al., 2007; Soria et al., 2003; Atherton et al., 1977; Addeo et al., 1983; Di Luccia et al., 1988; Eigel et al., 1984). The frequency of A allele is highest in many of the dairy cattle breeds, but the selection is intensively carried out against the B allele by selecting such cows and forming specific cattle herds that produce milk suitable for cheese production. A lot of focus is concentrated on the B allele, as it is associated with improvement of milk processing properties (Kübarsepp et al., 2005; Strzalkowska et al., 2002). Relatively high frequency of kappa-casein B allele has been found in the cattle of Italy, France, Germany and UK (0,400 – 0,840) (Jann et al., 2004), slightly lower in Turkish (0,2979 – 0,3452), Croatian (0,130 – 0,460), Polish (0,33) and Belgian (0,190 – 0,280) cattle (Jann et al., 2004) as well as in Lithuanian cows, as shown by the results of our study (0.227). High frequency of kappa-casein B allele has been determined for Estonian brown and Jersey breeds, while lower frequency - in Estonian

Holstein and Argentinian Holstein breeds (Bonvillani et al., 2000; Beja-Pereira et al., 2002; Kubarsepp et al., 2005).

Kappa-casein E allele has not been found in Italian (Caroli et al., 2004; Jann et al., 2004), French (Jann et al., 2004), Belgian (Jann et al., 2004), Croatian (Jannet et al., 2004) and Turkish cattle (Dinc et al., 2013). The frequency of E allele for the Lithuanian cattle was 0,052 as found in this study.

High frequency of A allele, as well as in our study, has been found in Chinese Holsteins and Simmentals (Trakovická et al., 2012), also among Holstein cattle, bred in India, Russian black and white and Russian brown, and Columbian Holstein breeds (Zmbrano-Burbano et al., 2012).

The importance of B allele and BB genotype of kappa casein for milk manufacturing properties is reported in several studies (Martin et al., 2002; Hoogendoorn et al., 2003; Caroli et al., 2009; Pauciullo et al., 2013). In dairy cattle the B variant of kappa casein is associated with a higher protein content, better quality of curd and increased yield of cheese. It has been suggested that identification of kappa casein genotypes could be an economically important selection criteria for dairy herds designated for industrial milk production (Pedersen, 1991).

Evaluation of actual and theoretical heterozygosity in the studied milk cattle population reared in Lithuanian revealed that theoretical heterozygosity was lower than the actual. The difference was not statistically significant, thus showing that, according to the Hardy-Weinberg principle, the studied group of cattle is in genetic equilibrium at the locus of kappa-casein gene. Observed higher than predicted actual heterozygosity indicates high level of genetic variation, which creates favorable conditions for livestock breeding.

Introduction of genetic markers into breeding programs allows to increase selection effectiveness and to expand the spectrum of traits, which can be effectively manipulated. The use of markers enables selection by a single trait only, which is determined by a single gene, or by several traits that are dependent on several genes. Consequently, with the rise of new molecular methods for genotype evaluation, new opportunities for cattle selection emerge (Miceikienė et al., 2007).

Conclusions

1. In this study, the polymorphism of milk protein gene kappa-casein was investigated in milk cattle reared in Lithuania. Three alleles with following frequencies A (0.72), B (0.23) and E (0.05) and six genotypes AA, AB, AE, BB, BE, EE were identified. The most frequent genotype AA was identified for 49.5% of tested animals. The least frequent genotype was EE, which was found only in 0.5% of investigated cattle.

2. BB genotype, which highly influences the processing properties of milk, was found in 2.1% of investigated cows.

3. Cows with kappa casein AA genotype had the highest genomic scores for milk yield (6.05), observing statistically significant difference between kappa casein

AA and AB genotypes ($P < 0.05$); highest protein amount genomic values had kappa casein BB genotype cows (6.25), highest protein percent genomic scores had BB and BE genotype cows.

4. Dispersion analysis showed that 2.8 % of milk yield variation expressed by genomic scores ($P > 0.05$), 0.2% of protein amount variation ($P > 0.05$) and 3.3 % of protein percent variation ($P < 0.05$) are dependent from kappa casein gene genotype.

5. The studied milk cattle population reared in Lithuania was in genetic equilibrium at the locus of kappa casein gene according to the Hardy-Weinberg principle. Observed higher than predicted actual heterozygosity indicates high level of genetic variation, which creates favorable conditions for livestock selection. Obtained results provide opportunities for selection of Lithuanian dairy cattle population in order to improve milk processing properties.

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